Title

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Is Predominant Clonal Evolution a Common Evolutionary Adaptation to Parasitism in Pathogenic Parasitic Protozoa, Fungi, Bacteria, and Viruses?

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Abstract

We propose that predominant clonal evolution (PCE) in microbial pathogens be defined as restrained recombination on an evolutionary scale, with genetic exchange scarce enough to not break the prevalent pattern of clonal population structure. The main features of PCE are (1) strong linkage disequilibrium, (2) the widespread occurrence of stable genetic clusters blurred by occasional bouts of genetic exchange ('near-clades'), (3) the existence of a "clonality threshold", beyond which recombination is efficiently countered by PCE, and near-clades irreversibly diverge. We hypothesize that the PCE features are not mainly due to natural selection but also chiefly originate from in-built genetic properties of pathogens. We show that the PCE model obtains even in microbes that have been considered as 'highly recombining', such as *Neisseria meningitidis*, and that some clonality features are observed even in *Plasmodium*, which has been long described as panmictic. Lastly, we provide evidence that PCE features are also observed in viruses, taking into account their extremely fast genetic turnover.

The PCE model provides a convenient population genetic framework for any kind of micropathogen. It makes it possible to describe convenient units of analysis (clones and near-clades) for all applied studies. Due to PCE features, these units of analysis are stable in space and time, and clearly delimited. The PCE model opens up the possibility of revisiting the problem of species definition in these organisms.

We hypothesize that PCE constitutes a major evolutionary strategy for protozoa, fungi, bacteria, and viruses to adapt to parasitism.

List of Abbreviations

AFLP	Amplified fragment length polymorphism
CNV	Copy number variation
LD	Linkage disequilibrium
MCI	Multiclonal infection
MLEE	Multilocus enzyme electrophoresis
MLG	Multilocus genotype
MLST	Multilocus sequence typing
PCE	Predominant clonal evolution
PFGE	Pulse field gel electrophoresis
RAPD	Random amplified polymorphic DNA
RD	Russian doll

RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
ST	Sequence type (MLST genotype)
WGS	Whole genome sequencing

1. INTRODUCTION

The population genetics of microbial pathogens (parasitic protozoa, fungi, bacteria and viruses) has strong implications in applied science, human and veterinary medicine, and agronomy. Moreover, it provides fascinating models to the evolutionists through specific features such as short generation times, huge population sizes, adaptation to parasitism, host–pathogen coevolution, among others.

It is regrettable that this field has suffered from a considerable compartmentalization among specialists. This has occurred even between specialists working on closely related organisms, such as *Trypanosoma* and *Leishmania*, or even African and American trypanosomes. The result is that common features have been occluded by this tendency of specialists working on different pathogens failing to make relevant comparisons with other models.

We have used the opposite strategy and have performed, for the first time to our knowledge, in-depth comparisons through the thorough analysis of more than 450 articles (not all listed here) among a large number of species of parasitic protozoa (25 species), fungi (9 species), bacteria (32 species), and viruses (23 species and categories) (Table 1).

This approach is justified for three reasons. First, pathogens pose similar problems and challenges when applied research is concerned: the needs are the same for defining units of analysis, delimitating strains and species, and tracking epidemiological spreads; second, although pathogenic bacteria and viruses are not traditionally classified as parasites, they are from an evolutionary point of view, and one can expect that adaptation to parasitism selects adaptive traits similar to those of eukaryotic microbes; third, only a comparative approach makes it possible to draw general patterns, and, at the same time, to evidence the specificities of each model.

This survey has uncovered striking similarities of population structure and evolutionary patterns among these micropathogen categories, although they are separated from each other by vast evolutionary differences. These similarities could be either ancestral or due to convergent evolution. They might represent common adaptive strategies to parasitism. Shapiro (2016)

Table 1 List of the species under study

Bacteria	Fungi	Parasitic protozoa	Viruses
Bacillus anthracis	*Aspergillus fumigatus*	*Cryptosporidium andersoni*	Adenovirus
Bacillus cereus	*Candida albicans*	*Cryptosporidium hominis*	Chikungunya
Bartonella bacilliformis	*Candida Dubliniensis*	*Cryptosporidium muris*	DENV
Bartonella henselae	*Candida glabrata*	*Cryptosporidium parvum*	Ebola
Bartonella quintana	*Cryptococcus gattii*	*Giardia intestinalis*	Echovirus-Enterovirus
Borrelia burgdorferi	*Cryptococcus Neoformans*	*Leishmania braziliensis*	HAV
Burckholderia pseudomallei	*Fusarium oxysporum*	*Leishmania infantum* complex	HBV
Campylobacter coli	*Penicillium marneffei*	*Leishmania guyanensis*	HCV
Enterococcus feacium	*Pneumocystis jirovecii*	*Leishmania killicki*	HEV
Escherichia coli		*Leishmania lainsoni*	HIV
Helicobacter pylori		*Leishmania major*	Influenza
Legionella pneumophila		*Leishmania mexicana*	Maize streak virus
Listeria monocytogenes		*Leishmania peruviana*	Measles virus
Mycobacterium bovis		*Leishmania tropica*	Picornavirus
Mycobacterium tuberculosis		*Plasmodium falciparum*	Poxvirus
Neisseria gonorrhoeae		*Plasmodium floridense*	RABV
Neisseria lactamica		*Plasmodium vivax*	ScoV (SARS)
Neisseria. meningitidis		*Toxoplasma gondii*	SIV
Pseudomonas aeruginosa		*Trypanosoma brucei*	SLCov
Pseudomonas syringae		*Trypanosoma brucei gambiense*	VARV
Salmonella enterica		*Trypanosoma brucei rhodesiense*	VZV
Salmonella typhi		*Trypanosoma congolense*	WNV

Table 1 List of the species under study—cont'd

Bacteria	Fungi	Parasitic protozoa	Viruses
Staphylococcus aureus		*Trypanosoma cruzi*	
Streptococcus mitis		*Trypanosoma evansi*	
Streptococcus oralis		*Trypanosoma vivax*	
Streptococcus pneumoniae			
Streptococcus pseudopneumoniae			
Streptococcus pyogenes			
Vibrio cholerae			
Vibrio parahaemolyticus			
Vibrio vulnificus			
Xanthomonas campestris			

DENV, dengue virus; *HAV*, hepatitis virus; *HBV*, hepatitis B virus; *HCV*, hepatitis C virus; *HEV*, hepatitis E virus; *HIV*, human immunodeficiency virus; *RABV*, rabies virus; *SARS*, severe acute respiratory syndrom virus; *VARV*, Variola virus; *VZV*, varicella-zoster virus; *WNV*, West Nile virus.

has noted that 'pathogens are more likely than free-living bacteria to undergo clonal expansions, due in part to their ecology and transmission dynamics'.

2. THE MODEL OF PREDOMINANT CLONAL EVOLUTION AND ITS LAST DEVELOPMENTS

The predominant clonal evolution (PCE) model proposed by us (Tibayrenc et al., 1990; Tibayrenc and Ayala, 2012, 2013, 2014a; b) includes several specific assumptions that deserve to be recalled, because they are frequently misunderstood or misquoted.

In this model, clonality is understood with a clear and simple definition, that is to say: strongly restrained genetic recombination. This definition is widely accepted by many authors working on general evolution as well as on population genetics and evolution of micropathogens, including protozoa, fungi, bacteria and viruses. Our claim that many authors accept this definition, and accept also the view that selfing/inbreeding is a particular case of clonality (see A Debate in the Debate: Unisex/Selfing/Inbreeding versus 'Strict' Clonality) is not based mainly on self-citations (Ramírez and Llewellyn, 2015; Rougeron et al., 2015), but rather on the thorough analysis of a large number of articles (Table 2). Many times, authors consider scarcity of recombination, clonality and asexuality as

Table 2 Definitions of clonality used by various authors

General evolution	Parasitic protozoa	Fungi	Bacteria	Viruses	All pathogens
Arnaud-Haond et al. (2007) [a]	Anderson et al. (2000) [b,c,e]	Badoc et al. (2002) [a,d]	Baker et al. (2010) [b]	Holmes (2009 [b], 2013) [a,b]	Buscaglia et al. (2015) [a]
Avise (2015) [c]	Andersson (2012) [a,b]	Bovers et al. (2008) [b]	Balloux (2010) [b]	Morel et al. (2011) [b]	Xu (2004) [b]
Awadalla (2003) [e]	Annan et al. (2007) [b,c]	Calo et al. (2013) [d]	Bessen (2010) [a]	Perales et al. (2015) [b]	
de Meeus et al. (2007b) [a]	Barnabé et al. (2011) [d]	Campbell and Carter (2006) [e]	Bobay et al. (2015) [d]	Simon-Loriere and Holmes (2011) [b]	
Maynard Smith et al. (1993) [b]	Barnabé et al. (2013) [b]	Campbell et al. (2005) [b,c]	Budroni et al. (2011) [b]		
Prugnolle and de Meeus (2008) [a,b]	Beck et al. (2009) [b,c,e]	Carriconde et al. (2011) [a,b,e]	Ch'ng et al. (2011) [b]		
Schurko et al. (2008) [e]	Birky (2009) [c]	Chaturvedi and Chaturvedi (2011) [a]	Chaudhuri and Henderson (2012) [b]		
	Branch et al. (2011) [e]	Chowdhary et al. (2011) [e]	Clermont et al. (2011) [b]		
	Buscaglia et al. (2015) [c]	Feretzaki and Heitman (2013) [d]	Coscollá et al. (2011) [b]		
	Chargui et al. (2009) [c]	Fraser et al. (2005) [a]	Dagerhamm et al. (2008) [e]		
	Chenet et al. (2012) [e]	Giraud et al. (2008) [d]	Dale et al. (2011) [b]		
	Cooper et al. (2007) [b]	Heitman (2010) [c]	Denamur et al. (2010) [b]		
	de Waele et al. (2013) [e]	Henk et al. (2012) [a,b]	Didelot and Falush (2007) [b]		

Downing et al. (2011) [c]
Duffy et al. (2013) [c,e]
Falk et al. (2015) [c,e]
Feretzaki and Heitman (2013) [c]
Flores-López and Machado (2011) [b]
Gatei et al. (2007) [b,e]
Gelanew et al. (2010) [d]
Griffing et al. (2011) [b,c]
Grigg and Sundar (2009) [c]
Heitman (2006) [b,c]
Herges et al. (2012) [e]
Iwagami et al. (2012) [e]
Karunaweera et al. (2008) [e]
Khan et al. (2011) [b,e]
Kuhls et al. (2008) [c,e]
Khayhan et al. (2013) [b,e]
Lin and Heitman (2006) [b]
McManus and Coleman (2014) [b]
Ngamskulrungroj et al. (2009) [b,e]
Ni et al. (2013) [b,c]
Taylor (2015) [b,c]
Xu (2006) [b,e]
Didelot (2010) [b]
Dos Vultos et al. (2008) [b]
Edwards et al. (2008) [e]
Fargier et al. (2011) [b,e]
Feil (2010) [b]
Fraser et al. (2007) [b]
Gomez-Valero et al. (2009) [b]
Guttman and Stavrinides (2010) [b,e]
Hanage et al. (2006) [a,b]
Henriques-Normark et al. (2008) [b,e]
Kurtenbach et al. (2010) [b]
Maiden (2006 [a], 2008) [b]
Martin et al. (2010) [e]
Pérez-Losada et al. (2006), (2013) [b]
Pirnay et al. (2009) [a,b]

(*Continued*)

Table 2 Definitions of clonality used by various authors—cont'd

General evolution	Parasitic protozoa	Fungi	Bacteria	Viruses	All pathogens
	Leblois et al. (2011) [b]		Prasad Narra & Ochman (2006) [a,b]		
	Lehmann et al. (2004) [c,e]		Robinson et al. (2011) [b]		
	Llewellyn et al. (2009a [d], 2009b, 2011 [b])		Sarkar and Guttman (2004) [b,e]		
	Lymbery and Thompson (2012) [c]		Sheppard et al. (2010) [b]		
	Minning et al. (2011) [a,b]		Smyth and Robinson (2010) [b,e]		
	Miotto et al. (2013) [c]		Supply et al. (2003) [e]		
	Morrison et al. (2008a [b,e], 2008b [b], 2009 [c])		Tenaillon et al. (2010) [b]		
	Mu et al. (2005) [b,c,e]		Vogel et al. (2010) [b,e]		
	Mzilahowa et al. (2007) [c,e]		Wiehlmann et al. (2007) [a,b,e]		
	Nkhoma et al. (2013) [b,c,e]				
	Orjuela-Sánchez et al. (2010) [e]				
	Rajendran et al. (2012) [b,e]				
	Ramírez et al. (2012) [e]				
	Razakandrainibe et al. (2005) [b,c]				
	Rezende et al. (2010) [e]				
	Rogers et al. (2014) [a,e]				

Rougeron et al. (2009, 2010, 2014, 2015 [d])
Sibley and Ajioka (2008) [b,c]
Smith (2009) [b]
Su et al. (2003) [a,b]
Su et al. (2006 [e], 2010 [b], 2012 [c])
Takumi et al. (2012) [b,e]
Tanriverdi et al. (2008) [b,e]
Thompson et al (2011) [b,c]
Tomasini et al. (2014) [e]
Volkman et al. (2007 [e], 2012a [e], 2012b [b,c,e])
Wang et al. (2012) [b,e]
Weedall and Hall (2014) [c,e]
Weir et al. (2016) [d,e]
Wendte et al. (2010 [c], 2011 [b])

[a]Clonality asexual reproduction.
[b]Clonality amounts to restrained recombination.
[c]Selfing/inbreeding are particular forms of clonality and are not distinct from it.
[d]Clonality is restrained to mitotic clonality and should be distinguished from selfing/inbreeding.
[e]Linkage disequilibrium is used for detecting restrained recombination.

synonymous terms (Hanage et al., 2006; Holmes, 2013). Including selfing/inbreeding in the general frame of clonality or distinguishing 'true' clonality (mitotic reproduction) from selfing/inbreeding is a matter of definition. We do not state that the second view should be definitely rejected. We only assert that the first view permits to enlighten highly relevant, common predictive properties and raises fewer problems of interpretation.

An important feature of the model, recalled many times by us, is that it does not state that recombination is totally lacking or that its evolutionary and epidemiological consequences are negligible (as wrongly understood by some authors: Calo et al., 2013; Messenger and Miles, 2015; Miles et al., 2009; Ramírez et al., 2012; Ramírez and Llewellyn, 2014), but only that it is not frequent enough to break up the predominance of clonal evolution (see further for a sharp definition of 'predominance/predominant').

Knowing whether populations of pathogens recombine freely or scarcely (PCE) has considerable implications for our knowledge of the basic biology of these organisms and for applied research (dynamics of genes of interest, strain typing, clinical research, vaccine and drug design). In case of abundant recombination, multilocus genotypes (MLGs) are ephemeral, since they are constantly disrupted by frequent genetic exchange. The evolutionary unit is not the MLG, but rather, the individual gene. When the organism undergoes PCE, MLGs are stable in space and time. They are the relevant evolutionary units. In sexual (recombining) organisms, there are no properly said 'strains' (stable MLGs).

Unambiguously, the PCE concept of clonality refers to genetic clonality, and not to any specific type of cytological mechanism. It includes, not only mitotic clonality but also all cases where recombination is rare or absent (Tibayrenc et al., 1990), such as several cases of parthenogenesis, gynogenesis and hybridogenesis (Avise, 2004, 2008, 2015), as well as self-fertilization in homozygous states, strong inbreeding and 'unisexuality' (Feretzaki and Heitman, 2013). As we have shown (Tibayrenc and Ayala, 2012, 2013, 2014a and b), this definition is widely used by researchers working on population genetics of all kinds of micropathogens (Table 2). It is also accepted by scientists working on unisexual vertebrates (Avise, 2004, 2008, 2015). Nevertheless, this definition is sometimes misunderstood, or not accepted by some researchers, who recommend distinguishing selfing/inbreeding from 'strict' clonality (see 'A debate in the debate: unisex/selfing/inbreeding versus "strict" clonality').

Based on this definition, the three main features generated by PCE are (1) strong (statistically significant) linkage disequilibrium (LD), not generated by physical obstacles (time and/or space isolation: the Wahlund effect); (2) strong phylogenetic signal evidencing the occurrence of stable, discrete genetic subdivisions, for which we have coined the term 'near-clade' (Tibayrenc and Ayala, 2012), the reason for which will be stated later; and (3) 'clonality threshold', beyond which recombination is efficiently countered by PCE, and the near-clades irreversibly diverge. Two additional properties of the PCE model are: (4) the repetition of MLGs that are overrepresented under panmictic expectations and (5) the propagation of stable MLGs over vast spans of time and space. Obviously, these different properties of the PCE model are linked to each other.

Another specific proposal of the PCE model is that restrained recombination is not 'passive', that is, due to lack of opportunity for mating (the so-called starving sex hypothesis; Tibayrenc and Ayala, 2014a,b). In the PCE model, restrained recombination is also not caused chiefly by natural selection acting on an otherwise recombining species, as it has been proposed for *Neisseria meningitidis* (Buckee et al., 2008). The PCE model hypothesizes that restrained recombination is a specific evolutionary strategy of micropathogens and is governed by their specific in-built properties (Tibayrenc and Ayala, 2002). PCE can be considered as the total set of reproductive strategies used by micropathogens to escape the 'recombinational load' (disrupting favourable multilocus associations; Agrawal, 2006; Beck and Agrawal, 2012; Butlin, 2012; Feretzaki and Heitman, 2013; Michod et al., 2008), probably as a major adaptation to parasitism.

Lastly, the PCE approach focuses more on those parts of the genome that will better evidence the overall phylogeny of the species, and its 'clonal backbone' (Sarkar and Guttman, 2004). In the case of eukaryotic microbes, it focuses on the nuclear genome rather than on the mitochondrial genome, and for bacteria, on the chromosomal DNA rather than on extra-chromosomal elements, and, within the chromosomal DNA, on the core genome rather than on the dispensable genome. Other genomic parts are placed onto this overall phylogeny/clonal backbone in a phylogenetic character mapping approach (Avise, 2004). As a matter of fact, these other genomic elements follow different evolutionary patterns, selective pressures and modes of inheritance. They are bound to yield phylogenies that are not congruent with the overall phylogenies of the DNA sequences targeted by the PCE approach, even in the absence of recombination.

We will now consider in more detail the main PCE features.

2.1 Strong (statistically significant) linkage disequilibrium

LD is the nonrandom association of genotypes occurring at different loci. It is the logical consequence of lack or scarcity of recombination. Its strength can be measured by various statistics, the most classical one being the *Ia* association index (Maynard Smith et al., 1993). We have proposed several other tests for evaluating LD (Tibayrenc et al., 1990). A specially telling case of LD is when the genetic distances measured with radically different molecular markers prove to be highly correlated (the 'g' test: Tibayrenc et al., 1990). Sometimes, when such a congruence between different genetic markers is strong, it can be visualized on gels (Fig. 1) or on phylogenetic trees (Fig. 2).

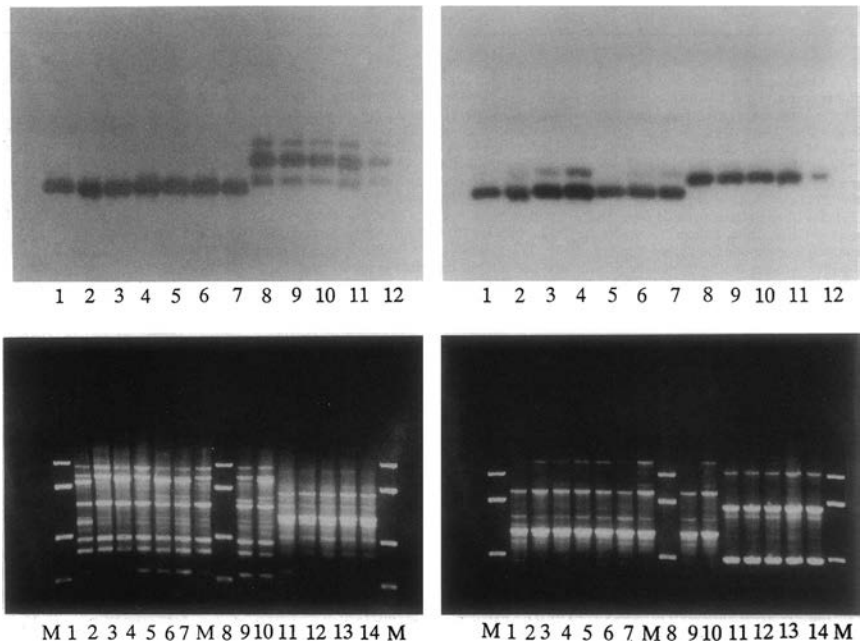

Figure 1 A striking case of linkage disequilibrium in *Trypanosoma cruzi*. Multilocus enzyme electrophoresis (top) and random amplified polymorphic DNA (bottom) are totally linked to each other. Cross-genotypes (for example: A1 with D10) have never been observed among more than 500 strains. *After Tibayrenc, M., Kjellberg, F., Ayala, F.J., 1990. A clonal theory of parasitic protozoa: the population structure of* Entamoeba, Giardia, Leishmania, Naegleria, Plasmodium, Trichomonas *and* Trypanosoma*, and its medical and taxonomical consequences. Proc. Natl. Acad. Sci. U.S.A. 87, 2414–2418.*

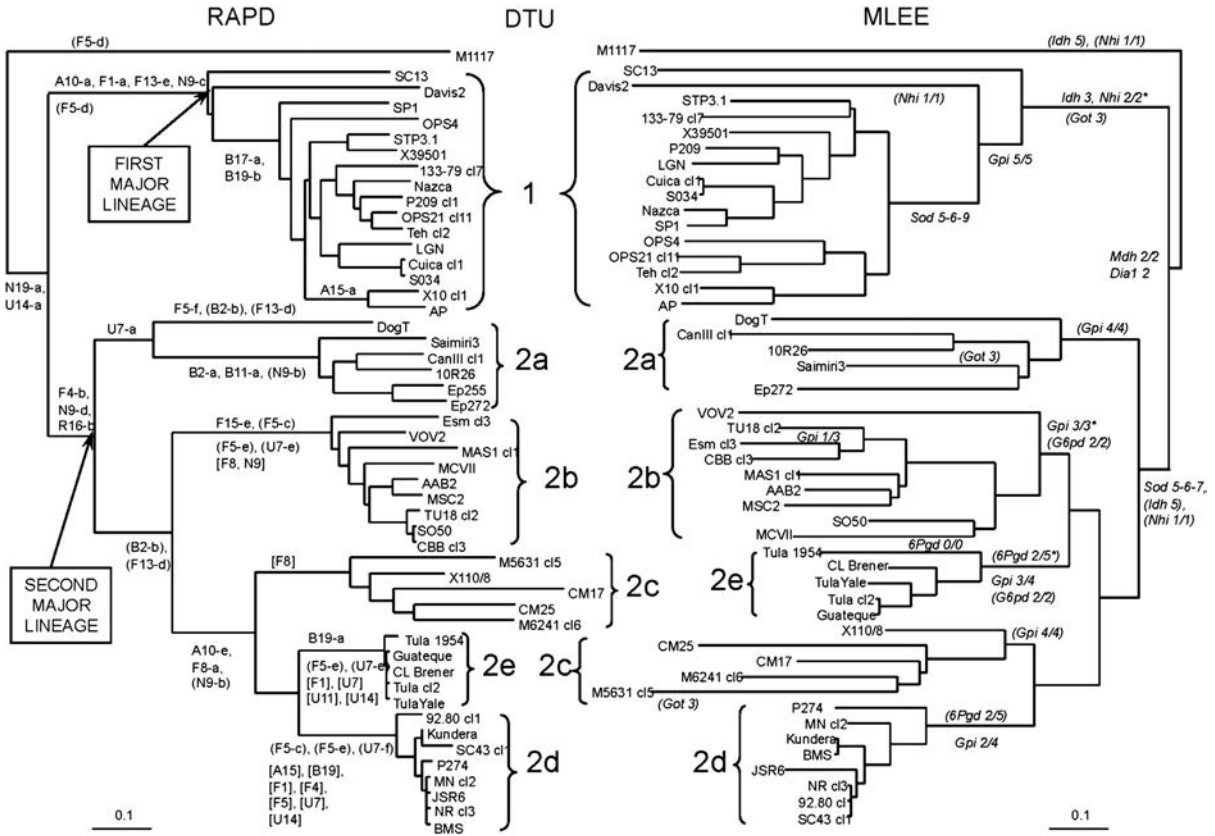

Figure 2 Double tree showing the close parity between random amplified polymorphic DNA (RAPD) (left) and multilocus enzyme electrophoresis (MLEE) (right) in *Trypanosoma cruzi*. The two kinds of markers uncover the same six near-clades or 'discrete typing units' (DTUs). Near-clade labelling has been changed (I to VI; Zingales et al., 2012). *After Brisse, S., Barnabé, C., Tibayrenc, M., 2000. Identification of six* Trypanosoma cruzi *phylogenetic lineages by random amplified polymorphic DNA and multilocus enzyme electrophoresis. Int. J. Parasitol. 30, 35–44.*

LD has been considered unreliable for exploring the reproductive strategy of pathogens for its supposed lack of resolution, as exposed in the case of *Leishmania* (Rougeron et al., 2010). However, (1) it is widely used by authors who explore clonality in parasitic protozoa, fungi, bacteria and viruses (Tibayrenc and Ayala, 2012; see Table 2); (2) it is the specific statistic designed for exploring lack or scarcity of recombination, the very definition of clonality used by us and by many others; and (3) when a sufficient number of variable loci is used, its resolution power is considerable. Our early proposals on clonal evolution in various eukaryotic microbes (Table 3) were mainly based on LD. Since many of these hypotheses have been further confirmed, this suggests that LD is not an unreliable statistics to explore the population structure of pathogens. Actually, LD and phylogenetic analysis can be considered as two complementary ways to explore the same phenomenon, that is to say: genetic isolation among evolutionary lines. Biases such as insufficient samplings and Wahlund effects are not limited to LD analysis, and also affect phylogenetic approaches.

2.2 Strong phylogenetic signal evidencing the occurrence of stable, discrete genetic subdivisions ('near-clades')

As we have insisted on, the PCE model does not imply that recombination is absent, but only that it is insufficient to erase the manifestations of PCE. Actually, it can be suspected that pathogens that are 100% clonal are very rare. The fact that some recombination most times goes on at various doses has two implications: (1) Pathogens' genetic subdivisions should not be named by the term 'clade', which, strictly speaking, designates evolutionary lines that are totally separated from each other. However, this term, which is most times improper in the context of micropathogen evolution, is widely used in the literature, in parasitic protozoa (Ramírez et al., 2012; Su et al., 2012), fungi (McManus and Coleman, 2014; Voelz et al., 2013), bacteria (Chaudhuri and Henderson, 2012; Croucher et al., 2011) and viruses (Liu et al., 2011; Raghwani et al., 2011). This is why we have coined the term 'near-clade' to designate genetic clusters of pathogens that are somewhat blurred by occasional recombination (Tibayrenc and Ayala, 2012). (2) As a linked proposal to (1), the occurrence of occasional bouts of recombination implies that a strict cladistic approach is not suitable to characterize the phylogenetic signal of the near-clades, even when it is strong. We have proposed (Tibayrenc and Ayala, 2012) to rather use a flexible phylogenetic approach based on a congruence criterion inspired from the principle of genealogical concordance between independent genes proposed for the

Table 3 Species analysed in Tibayrenc and Ayala (1991a, Table 2): rank of evidence for clonality

Organism	Rank	Criteria supporting clonality
Fungi		
Candida albicans	i	None
Candida tropicalis/Candida paratropicalis	ii	*d*1, *d*2, *e*, *f*
C. neoformans B + C serotypes	ii	*e*, *f*
C. neoformans A + D serotypes	ii	*f*
C. neoformans all serotypes	ii	*d*1, *d*2, *e*, *f*
Saccharomyces cerevisiae	ii	*f*
Protozoa		
Entamoeba histolytica	iii	*d*1, *d*2, *e*, *f*
Giardia sp.	iii	*d*1, *d*2, *e*, *f*, *g*
Leishmania braziliensis guyanensis	iv	*d*1, *d*2, *e*, *f*
Leishmania infantum	iv	*d*1, *d*2, *e*, *f*
Leishmania tropica	iv	*a*, *d*1, *d*2, *e*, *f*
Leishmania major	iv	*d*1, *d*2, *e*, *f*
Leishmania Old World as a whole	iv	*d*1, *d*2, *e*, *f*
Leishmania sp.	iii	*g*
Naegleria australiensis	ii	*a*, *d*
Naegleria flowleri	ii	*a*
Naegleria gruberi	ii	*a*
Plasmodium falciparum	ii	*d*1, *d*2, *e*, *f*, *d*
Toxoplasma gondii	ii	*d*1, *d*2, *f*
Trichomonas foetus	ii	*d*
Trichomonas vaginalis	ii	*d*
Trypanosoma brucei s.l.		
West Africa	iv	*d*1, *d*2, *e*, *f*
East Africa	iv	*d*1, *d*2, *e*, *f*
East Africa (wild)	ii	*e*, *f*
Liberia	iv	*d*1, *d*2, *e*, *f*
Busoga, Uganda	iv	*d*1, *d*2, *e*, *f*
Lambwe Valley, Kenya	iv	*d*1, *d*2, *e*, *f*
Lambwe Valley (nonhuman stocks)	iv	*d*1, *d*2, *e*, *f*
Ivory Coast	iv	*d*1, *e*, *f*
Ivory Coast (nonhuman stocks)	iv	*d*1, *f*
Trypanosoma brucei rhodesiense	ii	*a*, *d*1, *d*2, *e*, *f*
Trypanosoma congolense	iii	*a*, *d*1, *d*2, *e*, *f*
Trypanosoma cruzi	iv	*a*, *b*, *c*, *d*, *f*, *g*
Trypanosoma vivax	iv	*d*1, *d*2, *e*, *f*

i, the available data do not evidence clonality; ii, clonality is only a working hypothesis because the supporting evidence comes from small samples; iii, there is evidence for clonality but the limited number of markers prevents equating the strains with actual clones; iv, clonal population structure is well ascertained. Criteria supporting clonality are based on population genetics tests proposed by Tibayrenc et al. (1990). *a*–*c*, segregation tests (within loci), relying on Hardy–Weinberg equilibrium; *d*–*g*, recombination tests (between loci), relying on linkage disequilibrium analysis.

recognition of biological taxa (Avise and Ball, 1990; Avise, 2004). Sharp genealogical concordance between any two independent genes is too strict a criterion, since (1) within the PCE model, some recombination may occur and somewhat cloud the phylogenetic signal and (2) discrepancies between independent genes may occur for several reasons other than recombination, even in the case of different species (see further, 'Biases Towards Recombination or Clonality'). We rather recommend using the congruence criterion as follows. If the phylogenetic signal weakens with additional adequate data, this is an indication that recombination plays a major role in the micropathogen's population structure and efficiently counters the structuring of the species considered. This is the pattern described by the 'semiclonal model' (Maiden, 2006) and the 'epidemic clonality model' (Maynard Smith et al., 1993, Fig. 3).

If on the contrary, additional relevant data reveal a growing phylogenetic signal, the hypothesis of PCE is supported. This is what we have called the 'clonality threshold', beyond which clonal evolution becomes preponderant, that is, efficiently counters the impact of recombination and causes this growing phylogenetic signal. This clonality threshold concept is therefore neither 'pseudoquantitative' nor 'vague', and 'predominant' is not open to 'wide interpretation' either (Ramírez and Llewellyn, 2015). The clonality threshold relies on the observation of a growing phylogenetic signal, which is easy to verify with appropriate data (see later).

When bacteria are concerned, the similar concept of 'clonal/sexual threshold' states that beyond a given rate of recombination, divergence is inhibited, and clusters no longer diverge but are constantly reabsorbed into the parent population by the cohesive force of recombination (Fraser et al., 2007). This would correspond to the semiclonal model (Maiden, 2006) and the 'epidemic clonality model' (Maynard Smith et al., 1993). Below this threshold, or beyond the clonality threshold, divergence counters homogenization and PCE begins (Tibayrenc and Ayala, 2012). Clonal species are assumed to exhibit 'tree-like' phylogenies (Maiden, 2006). In contrast to the clonality threshold proposed by us, the clonal/sexual threshold model of bacteria (Fraser et al., 2007) is not based on the observation of a growing phylogenetic signal, but rather, on the recombination/mutation (r/m) ratio, which is subject to clashing data. As an example, r/m was estimated as 7.2 in a study dealing with *Streptococcus pneumoniae* whole genome sequencing (WGS) (Croucher et al., 2011), whereas Multilocus Sequence Typing (MLST) analysis (Maiden, 2006) had given a ratio of 66 (Feil et al., 2000). Vos and Didelot (2009), with a homogeneous

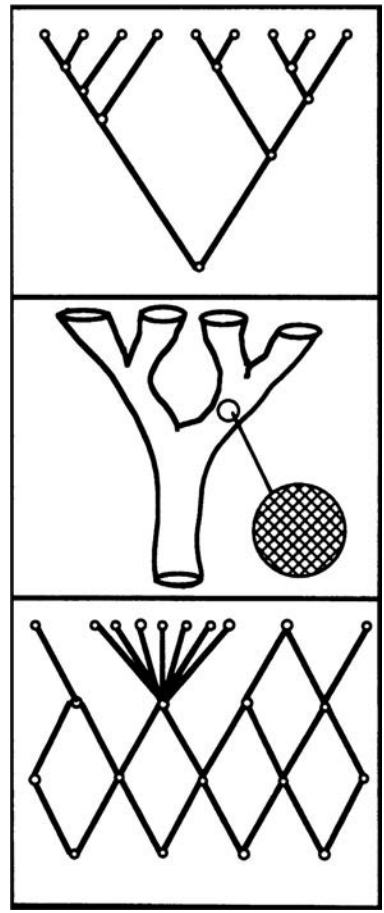

Figure 3 Models of pathogen population structures proposed by Maynard-Smith et al. (1993). Top, clonal evolution; middle, there is no genetic exchange between the two major branches, but recombination is frequent within each of them, leading to a network structure rather than to a tree-like structure; bottom, epidemic clonality.

methodology (MLST) analysed with the ClonalFrame method, have found r/m rates that are poorly compatible with the known biology of the species surveyed by them (see Table 1 in their article). *Salmonella enterica*, assumed to be highly clonal (Maynard Smith et al., 1993), has an r/m of 27.4–48.2; *Helicobacter pylori*, considered panmictic (Suerbaum et al., 1998) or 'almost non-clonal' (Henriques-Normark et al., 2008), has an r/m of 12.2–15.4; and *N. meningitidis*, presented as 'highly recombining' ('epidemic population structure'; Maynard Smith et al., 1993), has an r/m of 5.1–9.5. WGS in *Escherichia coli* shows that the rate of recombination is almost equal to the

rate of mutation. However, this high rate of recombination 'does not destroy the clonal status of *E. coli*' (Bobay et al., 2015). These results on recombination versus mutation rates suggest that they have a poor predictive power regarding the actual population structure of the species considered. It might be illusory to rely on such fallaciously sharp mathematical indices to explore the population structure of micropathogens. This is all the more true, because (1) near-clades should not be defined by precise levels of phylogenetic divergence, as it is the case for the genospecies concept in bacteria (Grimont, 1988), but only by their discreteness and stability in space and time and (2) pathogenic microbes are separated by vast evolutionary distances. They exhibit considerably diverse genomic structures and evolutionary patterns (Hupalo et al., 2015). Thus a common denominator in terms of mathematical estimation of the impact of recombination is probably unattainable. On the contrary, the clonality threshold and PCE features above exposed can constitute such a common denominator.

Sets of data relying on the congruence criterion for identifying near-clades and evidencing a growing phylogenetic signal could be, for example, based on our *g* test (Tibayrenc et al., 1990). If different kinds of genetic markers give congruent phylogenies, it is a clear indication of PCE. Data could be also based on different phylogenetic methods relying on different working hypotheses and giving congruent phylogenies, or the comparison between phylogenetic and non-phylogenetic methods (for example, STRUCTURE; Pritchard et al., 2000). If they show comparable clustering patterns, this is an evidence that phylogenies are robust, since they persist through various approaches based on different assumptions and evolutionary models. Other possible lines of evidence are adding more loci when multilocus enzyme electrophoresis (MLEE) is concerned (Brenière et al., 2003), or the phylogenies from different genes when MLST is used: phylogenies of individual genes could show discrepancies among them. However, the concatenated phylogeny based on the complete set of genes is strong and becomes stronger as other genes are considered (Diosque et al., 2014). Lastly, stability of the near-clades in time and space can be conveniently ascertained by retrospective studies, analysis of ancient collections and phylogeography/phylodynamics (Avise, 2000; Holmes, 2008; Holmes and Grenfell, 2009; Kenefic et al., 2010). This makes it possible to ascertain that near-clades are not the mere product of a Wahlund effect (isolation by distance and/or time; see further, 'Biases Towards Recombination or Clonality')

Near-clades are a remarkable consequence of strongly restrained recombination acting at an evolutionary scale. They have important implications in

terms of molecular epidemiology, taxonomy and applied research (see further, 'Relevance of the Predominant Clonal Evolution Model for Taxonomy and Applied Research'). In the examples given later, near-clades cannot be explained by mere geographical separation, even if this factor may contribute. As we will see later, near-clades have received many different names in the literature, leading to an extraordinarily confusing terminology (Table 4).

It has been argued that 'genetic subdivisions (i.e., near-clades) act as reproductive barriers', and therefore, that 'it makes little sense to address each parasite species (or genus) as a whole' (Ramírez and Llewellyn, 2014). For genera, we agree. For species, the PCE approach definitely proposes that the presently described species should be the starting point of analysis for exploring the pathogen's population structure. The existence of the near-clades within species is one of the major components of the PCE model. Establishing that near-clades are the results of reproductive barriers is the core of the PCE model, and one of its most important outcomes. 'A highly structured (e.g., clonal) population indicates that the main mode of reproduction for such a species lacks genetic exchange (is primarily asexual) or sex occurs only rarely' (Buscaglia et al., 2015). Considering that such a pattern was 'self-evident' (Ramírez and Llevellyn, 2015) is easy when arriving long after the battle and amounts to saying that the results of any phylogenetic analysis are self-evident. With such a view, the discovery of the newly described *Trypanosoma cruzi* near-clades Tc-Bat (Lima et al., 2015; Marcili et al., 2009; Pinto et al., 2012, 2016) was self-evident, as was the description of the lesser near-clades within the *T. cruzi* near-clade TCI (Guhl and Ramírez, 2011). Evidencing near-clades is all the less self-evident, since even the widely accepted number of near-clades within the thoroughly studied species *T. cruzi* (Brisse et al., 2000; Zingales et al., 2012) still is under debate (Barnabé et al., 2016). Lastly, it should be recalled that obstacles to gene flow and clonal evolution in various major species of eukaryotic microorganisms, including *T. cruzi*, *Trypanosoma brucei*, *Leishmania* sp., *Giardia intestinalis*, and *Toxoplasma gondii*, and the unexpected result of clonal propagation in *Plasmodium falciparum* have been proposed long before any within-species phylogenetic data were available for these species (Tibayrenc et al., 1981; 1986, 1990, 1991a; see Table 3). Such hypotheses were far from being self-evident. In a second time, convenient phylogenetic analyses were able to show that in *T. cruzi*, for example, obstacles to gene flow led to the individualization of six near-clades (Brisse et al., 2000). Unexpectedly, Ramírez and Llewellyn (2014) did not raise the problem of this 'artefactual

Table 4 The many different terms used in the micropathogen literature to designate pathogens' genetic clusters

Viruses	Bacteria	Parasitic protozoa	Fungi
Clades	Clades	Assemblages	Amplified fragment length polymorphism groups
Clusters	Clonal complexes	Clades	Clades
Genogroups	Clonal lineages	Clonal haplogroups	Clonal groups
Genotypes	Clonal subgroups	Clonal haplotypes	Clonal lineages
Groups	Clusters	Clonal lineages	Clusters
Lineages	Family strains	Clonal types	Clonal groups
Major genotypes	Genetic groups	Clones	Genetically distinct subgroups
Major lineages	Genoclouds	Clonotypes	Genotypes
Phylogenetic groups	Genogroups	Clusters	Genotypic groups
Phylogroups	Genome groups	Core subgroups	Groups
Subclades	Genospecies	Discrete typing units	Lineages
Subgenotypes	Groups	Divergent entities	Major clades
Subgenotype clusters	Lineages	Genetic groups	Minor clades
Subgroups	Major branches	Genetic types	Molecular genotypes
Sublineages	Major clusters	Genotypes	Molecular types
Substrains	Main/major lineages	Groups	Phylogenetic species
Subtypes	Major phylogenetic groups	Haplogroups	Subclusters
Subvariants	Phylogenetic clades	Haplotypes	Subgenotypes
Types	Phylogenetic groupings	Lesser subgroups	Subgroups
Variants	Populations	Lineages	Subpopulations
	Primary clusters	Main haplogroups	Varieties
	Principal genetic groups	Major clades	
	Secondary clusters	Major clonal lineages	
	Semi discrete lineages	Major groups	
	Subclones	Major monophyletic groups	
	Subclusters	Phylogenetic lineages	
	Subgroups	Populations	

Table 4 The many different terms used in the micropathogen literature to designate pathogens' genetic clusters—cont'd

Viruses	Bacteria	Parasitic protozoa	Fungi
	Sublineages	Subassemblages	
	Subpopulations	Subclades	
	Subspecies	Subclusters	
	Subspecies groups	Subgroups	
	Subtypes	Sublineages	
		Subpopulations	
		Subgenotypes	
		Subgroups	
		Subspecies	
		Subtypes	
		Subtype groups	
		Types	

This shows that the conceptual bases for describing them are quite uncertain, although they probably correspond to the same evolutionary entity ('near-clade').

approach' in the case of *T. gondii*, for which they accept the hypothesis of PCE based on data that consider the whole species (Sibley et al., 2009).

We are not claiming that we have been the only ones to describe clustering patterns within microbial species. On the contrary, the past and present developments of the PCE model are based on the analysis of a great deal of data published by other authors. However, describing clusters is one thing, whereas evidencing that they correspond to the precise evolutionary definition of near-clades is another thing and can be done only by confronting various sets of data, as we have done. Most times, the evolutionary status of the clusters described within microbial species is unclear or wrong, for example, when calling them 'clades'. This uncertainty is traduced by the extremely confusing terminology that these clusters have received (Table 4). This clearly shows that the conceptual bases for describing genetic clusters in micropathogens are disparate and fluctuating. The unifying concept of 'near-clade' would be welcome to get rid of this semantic Babel tower.

The ambition of the PCE model is not to flatly corroborate the existence of within-species clusters in microbes, but rather to give a common conceptual framework and precise definitions, to many disparate data and approaches.

The literature dealing with population genetics of micropathogens is also made confusing by the widespread use of vague, subjective expressions for evaluating the respective impact of clonality and recombination, such as

'far from being a clonal species' (Coscollá et al., 2011), 'extensive genetic exchange' (Caugant and Maiden, 2009), 'gross' incongruences (between phylogenetic trees) (Messenger et al., 2012), 'widespread genetic exchange' (Ramírez et al., 2012; Ramírez and Llewellyn, 2014), 'intense lateral exchange of genetic information' (Ramírez and Llewellyn, 2015), 'genetic exchange is a fairly common phenomenon' (Lima et al., 2014), 'genetic exchange playing a major role in *T. cruzi* population structure' (Minning et al., 2011), and others. It is impossible to totally discard such expressions in the scientific literature. However, they are misleading and poorly informative. The criteria above exposed, that is strong (statistically significant) LD and congruent data leading to a growing phylogenetic signal and a clonality threshold (near-clade pattern), make it possible to replace these imprecise expressions with a precise definition based on a clear-cut cursor marking the PCE threshold. It could be proposed that expressions such as 'widespread genetic recombination' be restrained to those situations where PCE can be clearly rejected (panmixia, or semiclonality/epidemic clonality).

Two other informative PCE features, tightly linked to each other, are the following.

2.3 Repeated multilocus genotypes that are overrepresented under panmictic expectations

It is a classical manifestation of clonality and a logical consequence of LD. However, if the marker considered has a fast molecular clock, and generates much diversity in a short time, this feature may not be observed — all individuals would have a different MLG even in the case of PCE (Arnaud-Haond et al., 2005; Seridi et al., 2008). As we will see further ('Identical Multilocus Genotypes is a Relative Notion'), this notion of repeated genotypes strongly depends on the mutation rate of the marker considered.

2.4 Propagation of stable multilocus genotypes over vast spans of time and space

Provided that the warning about the marker's mutation rate just exposed is considered, this is a strongly telling manifestation of PCE, with considerable epidemiological implications. Some pathogen MLGs behave like 'superspreaders', and are encountered, not only locally but also over continents and for tens of years.

We have advanced additional considerations that complete the PCE model, namely, the 'Russian doll (RD) model', and the 'starving sex model'.

2.5 The 'Russian doll' model (Tibayrenc and Ayala, 2013)

We have forged this concept for the case when one or the two main PCE features (LD and near-clading) are obtained, not only at the level of the whole species but also within the near-clades that subdivide it (Fig. 4).

This is evidence that the populations within these near-clades undergo PCE too. They are beyond the clonality threshold, where recombination is insufficient to counter PCE. Some authors indeed have proposed that apparent clonality in a given species could be due to the fact that recombination is inhibited between the clusters that subdivide this species, but not or very little within them (Campbell et al., 2005). The population structure within the clusters would be network-like rather than tree-like, and there would be no LD within them (Maynard-Smith et al., 1993, Fig. 3). The clusters would be similar to biological species, and clustering/LD at the level of the whole species would be due to this presence of these unknown recombining subdivisions within it. This is the very alternative (null

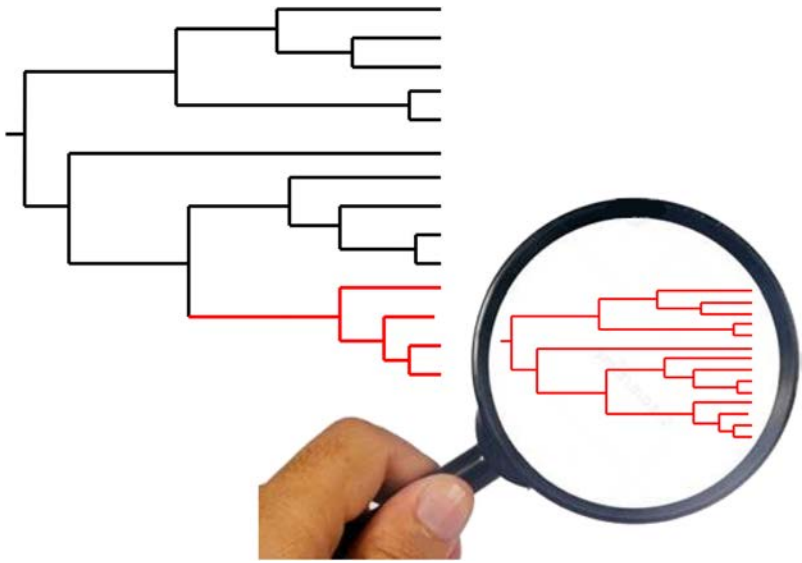

Figure 4 'Russian doll' model. When population genetic tests are practiced with adequate markers (of suitable resolution) within each of the near-clades that subdivide the species under study (large tree, left part of the figure), they evidence a miniature picture of the whole species, with the two main predominant clonal evolution features, namely, linkage disequilibrium and lesser near-clades (small tree, right part of the figure). This is evidence that the near-clades are not cryptic recombining entities (see Fig. 3, middle), and that they also undergo predominant clonal evolution.

hypothesis) to the RD model, which is definitely not falsified by occasional recombination occurring without erasing PCE features within the near-clades (Ramírez and Llewellyn, 2015). As we have insisted (Tibayrenc and Ayala, 2013), when exploring PCE within each near-clade, it is important to adapt the resolution of the markers and to carefully consider population sizes. As a matter of fact, if one changes evolutionary scales, apparent recombination could be due to a statistical type II error because the marker has too low a resolution power or because the sample size is insufficient, or both. Many examples aimed at challenging the RD model either rely on improper samples or denote a miscomprehension of the model (Ramírez and Llelwellyn, 2014). This model indeed has been considered as largely relying on insufficient samples and being an 'unnecessary oversimplification' (Ramírez and Llewellyn, 2015). However, all models are based on simplifications of diversified situations in which a common denominator can be evidenced. So are the RD and the PCE models, which rely on sharply defined, highly falsifiable, working hypotheses. The RD model is convenient for exploring within-near-clade population structure. Abundant examples with convenient sampling sizes supporting the model, in parasites, fungi, bacteria and viruses, will be exposed later.

2.6 The 'starving sex' model

It has been proposed that signs of clonality in *P. falciparum*, a parasite that had been long considered as panmictic (Walliker, 1991), were explained, not by intrinsic biological properties of the parasite, but rather, by the scarcity of multiclonal infections (MCIs) in low transmission cycles, leading to a lack of opportunity for different MLGs to mate and therefore, forcing the parasite to inbreed (Anderson et al., 2000). We have called this hypothesis the 'starving sex' model (Tibayrenc and Ayala, 2014a).

2.7 Biases towards recombination or clonality

Some biases can generate artefactual negative LD results, and may also yield clashing phylogenetic reconstructions. These are (1) homoplasy ('loci mutationally saturated'; Pearson et al., 2009b), which can be very important for microsatellites (Lehmann et al., 2004; Messenger et al., 2012; Schönian et al., 2010; Sibley and Ajioka, 2008), although Koffi et al. (2015) consider its impact as 'insignificant'; (2) different selective pressures; (3) different molecular clocks; and (4) different modes of genetic inheritance (mitochondrial versus nuclear genome).

On the contrary, the Wahlund effect (separation by physical obstacles: space and/or time) may restrain recombination in separate populations mistakenly pooled together and mimic the effects of PCE, a bias thoroughly exposed by Koffi et al. (2015) and Rougeron et al. (2014, 2015). The Wahlund effect may operate at microgeographic scales (Rougeron et al. (2014, 2015) and has certainly to be carefully taken into account in population genetic surveys. At macroscales of space and time, the ones that are considered by the PCE model, the Wahlund effect can be evidenced by showing that (1) overrepresented MLGs tend to be restricted to different geographical areas and/or different times (Tibayrenc et al., 1991a) and (2) near-clades tend to be linked to space and/or time, which can be evidenced by correlation between genetic and geographical distances, or a link between genetic distance and time. If on the contrary, MLGs and near-clades are persistent and widespread over vast geographic areas and spans of time, this supports the hypothesis that they are caused, not by the Wahlund effect, but rather, by in-built properties of the organisms under survey. It is difficult to understand how local Wahlund effects could lead to the same pattern over many years, different countries and continents, with the same MLGs, the same near-clades and RD patterns (Tibayrenc and Ayala, 2012, 2015d). The occurrence of stable near-clades and RD patterns in close sympatry, including in the same host, with therefore ample opportunity for genetic recombination, is a strong argument against the hypothesis that they are caused by a Wahlund effect (see further the case of *T. cruzi*). As a counter-example, the genetic subdivisions evidenced within *T. brucei gambiense* I by Koffi et al. (2015) are obviously linked to geographical distance, and could be equated to near-clades only if they are both observed together in sympatry or are recorded each over different countries in further studies.

The PCE model has been quoted 'artefactual', 'conceptually outdated', 'unnecessarily complicated' (Ramírez and Llewellyn, 2014, 2015), and 'challenged' (Messenger et al., 2012; Messenger and Miles, 2015; Ocaña-Mayorga et al., 2010; Rougeron et al., 2010). However, a model can be considered outdated or challenged only when it has been clearly rejected, not the way it is misleadingly enunciated by its opponents, but rather, as it is presented in the very terms (as mentioned earlier) of its authors. We argue that it is not the case for the PCE model, which is easy to understand, as we have been able to verify it with many non-specialists. It relies on the direct observation of abundant data that can be easily verified by anybody, the main ones being presented later (see also Tibayrenc and Ayala, 2012, 2013, 2014a,b, 2015a–d).

3. EVIDENCE FOR PREDOMINANT CLONAL EVOLUTION FEATURES IN VARIOUS KINDS OF MICROPATHOGENS

3.1 Parasitic protozoa

Examples selected in this part will focus on *T. cruzi*, the agent of Chagas disease; *G. intestinalis*; *T. gondii* and *P. falciparum*. A few other models will be more briefly considered.

T. cruzi is a paradigmatic case of PCE. Population genetic interpretation of MLEE variability made it possible to show that the 'zymodemes' (MLEE MLGs) evidenced by Miles' pioneering studies (1978) could be equated to genetic clones (Tibayrenc et al., 1981, 1986). In this species, there is LD: (1) among MLEE loci (Tibayrenc et al., 1981, 1986) and (2) between different kinds of markers: nuclear and mitochondrial polymorphisms (de Freitas et al., 2006; Machado and Ayala, 2001; Ramírez et al., 2011; Spotorno et al., 2008), random amplified polymorphic DNA (RAPD) and MLEE (Tibayrenc et al., 1993; Brisse et al., 2000), microsatellite polymorphism and DNA content (Lewis et al., 2009), neutral markers and strongly selected antigen genes (Lima et al., 2012; Rozas et al., 2007), as well as neutral genetic markers and the protein polymorphism revealed by proteomic analysis (Telleria et al., 2010). Although Minning et al. (2011) considered that their data suggested 'genetic exchange playing a major role in *T. cruzi* population structure', in their study, there is an obvious LD between CNV polymorphism and near-clade classification: all near-clade TCI strains clearly group together, as do all non-TCI strains (see their Fig. 3). This pattern is compatible with some genetic exchange going on, although other explanations are quite plausible (see 'Biases Towards Recombination or Clonality'). However, the overall tendency is that CNV polymorphism is linked to near-clade structuring. The same pattern of linkage between CNV polymorphism and near-clading has been observed by Reis-Cunha et al. (2015). Ubiquitous MLGs are very frequent in *T. cruzi* (Tibayrenc et al., 1986; Zingalez et al., 2012; Tibayrenc and Ayala, 2012, 2013). *T. cruzi* presents one of the most demonstrative cases of near-clading in trypanosomatidae, as well as one of the most clear among pathogens in general. The species is subdivided into six near-clades (known as 'discrete typing units' or DTUs) (Brisse et al., 2000; Zingales et al., 2012) (see Fig. 2). These near-clades are believed to have undergone hybridization processes, the history of which is still under dispute (Westenberger et al., 2005; de Freitas et al., 2006). It is widely accepted (Zingalez et al., 2012) that the near-clades TC V and VI have a

hybrid origin. They propagate themselves clonally, and are strongly associated to 'domestic cycles'. Hybridization in *T. cruzi* might be linked to adaptation to human environments and the conquest of new ecological niches, a phenomenon considered as widespread in parasites (King et al., 2015). All *T. cruzi* near-clades are very stable in space (from the United States to Argentina and Brazil), and time. They have been corroborated by many genetic markers, present some ecological and epidemiological specificities and exhibit differential protein expression patterns (Machin et al., 2014; Telleria et al., 2010). Interestingly, the six near-clades can be also discriminated by antigen genes (Rozas et al., 2007), although these genes undergo a strong selective pressure. This observation illustrates the strength of PCE in *T. cruzi*, which is reflected in all genes of this parasite, including strongly selected ones. An additional near-clade, referred to as Tc-Bat (because it has been isolated from bats only), has been recorded in Brazil, Colombia, Ecuador and Panama years apart, in different species of bats (Lima et al., 2015; Marcili et al., 2009; Pinto et al., 2012, 2016). This is a striking illustration of the permanency of the near-clades. The partitioning into seven near-clades has been questioned by Barnabé et al. (2016) on a vast sample of strains, but with a limited set of one nuclear gene and two mitochondrial genes. This proposal has obviously to be tested with a broader set of genetic markers. However, it shows that even in the extremely well-studied species *T. cruzi*, evidencing near-clades is far from being 'self-evident' (Ramírez and Llewellyn, 2015). It has been suggested that genetic isolation among *T. cruzi* near-clades could be due to ecological separation rather than due to in-built biological properties of the parasite (Messenger and Miles, 2015). Ecological separation might play a role. However, there are many instances of MCIs with two or more near-clades within the same chagasic patient and the same insect vector, providing ample opportunity for mating (Tibayrenc and Ayala, 1988). Within the near-clade TCI, illustrative RD patterns can be observed (Tibayrenc and Ayala, 2013). As a matter of fact, additional near-clades within TCI, which are stable, widespread and sometimes sympatric, have been labelled a to e (Guhl and Ramírez, 2011). They have been corroborated by various markers (Cura et al., 2010; Ramírez et al., 2011). These lesser near-clades present some pathogenicity specificities (Llewellyn et al., 2009b). They are statistically linked to geographical distance (Llewellyn et al., 2009b); however, they cannot be explained by a mere Wahlund effect, since, they have been recorded over vast geographical distances (Guhl and Ramírez, 2011). Additionally, strong LD and widespread clonality have been recorded in TCI (Llewellyn et al., 2009b, 2011). TCI

substructuring (RD pattern) in Northern Argentina ('Chaco 1-4') has been corroborated by analysis of the miniexon gene and MLST (Tomasini et al., 2014). A few studies have challenged an RD pattern within *T. cruzi* near-clades and are consistent with the hypothesis that recombination is frequent within TCI (Barnabé et al., 2013; Messenger et al., 2012; Ocaña-Mayorga et al., 2010) and TCII (de Paula Baptista et al., 2014). However, studies by Barnabé et al. (2013) and Ocaña et al. (2010) were based on limited samples (79 strains partitioned into six populations in the first one, 16 strains in the second one), and have to be confirmed by broader sets of data to eliminate a possible statistical type II error. In the study by Messenger et al. (2012), evidence for recombination was based on discrepancies between trees designed from mitochondrial and nuclear genotypes. The two categories have different modes of inheritance, and mix neutral (miniexon, microsatellites) and selected genes (coding mitochondrial genes). The data are compatible with mitochondrial introgression and occasional hybridization, although other explanations could be explored (see 'Biases Towards Recombination or Clonality'). They definitely do not reject the hypothesis of a PCE within TCI. In the study dealing with TCII, there is a contradiction between the apparent lack of LD and the evidence for a strong structuration of the populations, as shown by the STRUCTURE test by the authors themselves (de Paula Baptista et al., 2014). Moreover, constant positive *F*is values are at odds with the Hardy–Weinberg equilibrium inferred by the authors. One study inferring that recombination is frequent in TCI Colombian populations (Ramírez et al., 2013) is based on a strong misinterpretation. As a matter of fact, the authors claim that the populations exhibit linkage equilibrium, whereas the p values for linkage analysis are 4×10^{-4} (LD test) and 0.037 (*Ia* index of association), hence quite significant. Moreover, the hypothesis of linkage equilibrium in this population is at odds with the presence of two clearly delimited near-clades (RD pattern) supported by significant bootstrap values (Ramírez et al., 2013). This bootstrap result illustrates the usefulness of the flexible phylogenetic approach based on the congruence criterion used in the framework of the near-clade model. The authors have identified incongruences among trees of individual genes. However, in the final tree based on all genes, the near-clade pattern is quite clear.

We are not arguing that RD patterns are always verified within *T. cruzi* near-clades. However, they have been observed in several studies based on reliable samples and diversified genetic markers. Within-near-clade population genetics is just at its start in *T. cruzi* as well as in other

pathogens. The RD model gives a convenient working hypothesis to explore it.

Data are also very strong and complete in *G. intestinalis*, for which we have proposed that clonal evolution was observed (Tibayrenc et al., 1990, 1991a). Evidence for strong LD between different kinds of markers is abundant in this species (Feng and Xiao, 2011; Monis et al., 2009), with 'assemblages', that are perfectly equatable to near-clades (Tibayrenc and Ayala, 2014b). *Giardia* assemblages (labelled A to G) exhibit a neat, but not strict, host specificity and some phenotypic differences, and are corroborated by MLEE and gene sequences (Cacciò and Ryan, 2008; Feng and Xiao, 2011; Lasek-Nesselquist et al., 2009; Lebbad et al., 2011; Monis et al., 2009; Ortega-Pierres et al., 2009; Plutzer et al., 2010; Takumi et al., 2012; Xu et al., 2012). Additional subdivisions ('subassemblages') can be identified within the assemblages. These subassemblages (RD patterns) have been corroborated by many authors on various populations (Cacciò and Ryan, 2008; Feng and Xiao, 2011; Monis et al., 2009; Ortega-Pierres et al., 2009; Wielinga et al., 2011). They have been labelled A1 and A2 within assemblage A, and B3 and B4 within assemblage B. There are indications for subdivisions in the other assemblages (RD pattern) (Cacciò and Ryan, 2008; Feng and Xiao, 2011; Monis et al., 2009; Ortega-Pierres et al., 2009; Plutzer et al., 2010; Wielinga et al., 2011). The only example cited by Ramírez and Llewellyn (2015) to challenge the RD model in *Giardia* (Cooper et al., 2007) actually concerns not a whole assemblage (near-clade) but rather the subassemblage A2, whose existence is itself an evidence for an RD pattern. Moreover, it deals with the discrepancy of the phylogenies of only three genes and six strains. Although the data are compatible with recombination, (1) they say nothing about the frequency of recombination and (2) other explanations are equally possible (see 'Biases Towards Recombination or Clonality'). With very comparable data (discrepancies between sequences inferred from different genes in *Giardia*), Wielinga and Thompson (2007), rather than attributing them to recombination, invoked homoplasy and differences of molecular clock.

T. gondii, an apicomplexa parasite, besides strong LD (Khan et al., 2011; Su et al., 2012), shows typical near-clading patterns (Tibayrenc and Ayala, 2012, 2014a). This parasite, due to the classical notion of an 'obligatory' sexual cycle, like *P. falciparum*, has long been considered as panmictic (as recalled by Grigg and Sundar, 2009). However, we have proposed that clonality is present in *T. gondii*, and perhaps predominant (Tibayrenc et al., 1991a). This has been corroborated by further studies (Sibley and

Boothroyd, 1992). The three main clonal lineages recorded by various authors (Beck et al., 2009; Boothroyd, 2009; Rajendran et al., 2012; Sibley and Ajioka, 2008; Sibley and Boothroyd, 1992; Smith, 2009; Su et al., 2010), which predominate in European and North American domestic cycles, correspond to typical near-clades (Tibayrenc and Ayala, 2014a), corroborated by various markers and software. Clustering of antigen genes parallels that of introns (Khan et al., 2011). Remarkably, the phylogeny of the ROP 18 gene, which undergoes strong natural selection, is identical to the overall phylogeny of the species (Khan et al., 2009). The three clonal lineages exhibit phenotypic specificity (virulence) (Boothroyd, 2009; Sibley and Boothroyd, 1992). Additional lineages assimilable to near-clades have been recorded in other cycles and regions of the world (Dubey et al., 2011; Khan et al., 2011; Mercier et al., 2010, 2011). A classical notion has emerged, namely, that *T. gondii* undergoes more recombination in South America than in North America and Europe (Lehmann et al., 2004, 2006; Su et al., 2010). However, this view needs being nuanced. Indeed, high-resolution typing of no less than 956 strains collected worldwide based on PCR—restriction fragment length polymorphism (RFLP), intron sequences of housekeeping genes and microsatellites reveals that this parasite's overall population structure (including South America) exhibits deep phylogenies and is composed of 6 'major clades' (near-clades) (Su et al., 2012). Among the six clades described by Su et al. (2012), three of them (clades A, B and F) exhibit RD patterns, with LD and additional near-clades within each of them (Tibayrenc and Ayala, 2014a). An RD pattern is also observed within the near-clade 'haplogroup 12', with strong LD and overrepresented MLGs (Khan et al., 2011). Lastly, ubiquitous MLGs are very frequent in *T. gondii* (see for review Tibayrenc and Ayala, 2014a).

P. falciparum, the agent of the most malignant form of malaria, obviously constitutes a specific case in our survey. It has long been considered as panmictic (Walliker, 1991). However, we have proposed (Tibayrenc et al., 1990, 1991a; Ben Abderrazak et al., 1999; Urdaneta et al., 2001) that this parasite could undergo some kind of uniparental propagation. Later, the hypothesis of panmixia has been considered 'oversimplified' (Heitman, 2006). Indeed, *P. falciparum* shows strong cases of LD (Tibayrenc et al., 1990; Anderson et al., 2000; Ben Abderrazak et al., 1999; Urdaneta et al., 2001; Tibayrenc and Ayala, 2012, 2014a). *P. falciparum* shows many examples of overrepresented, ubiquitous MLGs (Tibayrenc and Ayala, 2014a). Identical *P. falciparum* MLGs have been recorded in different patients in various countries. Persistent MLGs have been recorded for up to 8 years

(Nkhoma et al., 2013). The same MLG has been found in Bolivia in 1994 and in Brazil in 1997–98 (Anderson et al., 2000). *P. falciparum* also shows indications of structuration and tendency for near-clading (Tibayrenc and Ayala, 2012, 2014a). However, these genetic subdivisions definitely cannot be equated to typical near-clades, since they are unstable and change over time (Griffing et al., 2011), although they can persist for up to 5 years (Branch et al., 2011). Despite the fact that they are more labile than typical near-clades, these clusters introduce a major stratification feature in *P. falciparum* natural populations. This should be taken into account in all studies dealing with the analysis of this parasite's relevant characters. Within these labile near-clades, LD is observed (Griffing et al., 2011), suggesting a nascent, probably unstable RD pattern. In West Cambodia, *P. falciparum* has been considered as 'essentially clonal' (Miotto et al., 2013). In this region, three clearly differentiated, sympatric subpopulations have been stable in four different sites for 5 years (Miotto et al., 2013). In conclusion, although *P. falciparum* does appear to be capable of clonal propagation, as proposed earlier (Tibayrenc et al., 1990, 1991a), leading to a clear genetic structuration of many populations of this parasite, it does not fit the main features of the PCE model.

We will now briefly consider other examples in parasitic protozoa.

Among *apicomplexa parasites, Plasmodium vivax* exhibits a very similar pattern to that of *P. falciparum*. It also shows clear cases of LD (see for review Tibayrenc and Ayala, 2014a). As in *P. falciparum*, labile near-clades are observed in *P. vivax*. In South Korea, two dominant groups persist in spite of high variability and a high rate of MCIs (Iwagami et al., 2012), which is at odds with the starving sex hypothesis (see further). In India, 3 clusters, not linked to geographical distance, are corroborated by Neighbour Joining (NJ), STRUCTURE, and PCA (Gupta et al., 2012). *P. vivax* population structuring is also recorded in Brazil (Orjuela-Sánchez et al., 2010; Rezende et al., 2010). Lability of genetic subdivisions in *P. falciparum* and *P. vivax* is a manifestation of the neat impact of genetic recombination in these parasites. However, the presence of this structuring pattern is one of the clear indications for clonal propagation in them and permits to definitely falsify the 'panmictic prejudice' (Tibayrenc et al., 1990, 1991a; Tibayrenc and Ayala, 2002, 2014a).

In the lizard parasite *Plasmodium floridense*, Falk et al. (2015) have attributed to PCE the existence of 11 genetic subdivisions (near-clades) tentatively equated to new species by the authors.

In *Cryptosporidium andersoni*, one MLG has been sampled in the United States, Canada and the Czech Republic (Feng et al., 2011). Another one

has been sampled in several Chinese provinces (Wang et al., 2012). In the species pertaining to the genus *Cryptosporidium*, some indications for near-clading are present. However, the data are not sufficient to strongly ascertain it (Tibayrenc and Ayala, 2014b). In *Cryptosporidium hominis*, four 'subtype groups' have been described (Gatei et al., 2007). Subtypes/clusters in *Cryptosporidium muris* and *C. andersoni* are supported by microsatellites, minisatellites and protein coding genes (Feng et al., 2011). They can be widespread and observed in different host species (Wang et al., 2012). Substructuring has also been observed in *Cryptosporidium parvum* (Ortega-Pierres et al., 2009). Population genetic studies should be completed in *Cryptosporidium* parasites. However, available data, although they suggest that clonal propagation is present in some populations, seem to not fit the PCE criteria (Tibayrenc and Ayala, 2014b).

Concerning kinetoplastid parasites, we have proposed (Tibayrenc et al., 1990, 1991a) that several species of the genus *Leishmania* undergo clonal evolution. A lot of recently obtained data support this hypothesis.

The *Leishmania braziliensis/Leishmania peruviana* complex exhibits near-clades that are corroborated by (1) the splitstree and STRUCTURE software and (2) MLEE, MLST and pulse field gel electrophoresis (PFGE) (Odiwuor et al., 2012).

In *Leishmania donovani* (included in the *Leishmania infantum* complex), there is a strong LD between single nucleotide polymorphisms (SNPs) and copy number variations (CNVs) (Downing et al., 2011; Minning et al., 2011).). In the same species, high-resolution typing by WGS/SNP of 204 clinical isolates from Nepal and India (Imamura et al., 2016) reveals a remarkable case of RD pattern. The strains are split into three distinct genetic lines. The main one, the 'core group', is composed of 191 strains. Remarkably, this group exhibits no polymorphism by microsatellite typing. However, SNPs, in this group, which represents a very small subsample of the whole *L. donovani* species, reveal a highly structured diversity at the microevolutionary scale. Six congruent monophyletic groups, supported by high bootstrap values, can be distinguished, supported by both maximum likelihood and model-based clustering (near-clades). One of these groups (ISC5) exhibits a lesser subgroup. Apart from these six monophyletic groups, eight hybrid lineages are observed. However, these hybrid lines give no indication for recombination within this core group. LD is recorded within each of the six monophyletic groups, which exhibit a strong stability in space and time: most of the monophyletic groups were recorded from 2002 to 2011, and many of them were sampled both in Nepal and India. All these

genotypes have a recent origin: the most common recent common ancestor of the core group originates from the middle of the 19th century, whereas the ISC2 and ISC 4–6 are as recent as the year 1960. This definitely shows a clear RD pattern with lesser near-clades and LD at this microevolutionary level. This shows also that the 'measurably evolving pathogen' approach (Biek et al., 2015) can now be applied to eukaryotic micropathogens: the epidemiology of these organisms can be followed in recent times through WGS and time-serial samplings.

LD between different genetic markers in the *L. infantum* complex is clear (Gouzelou et al., 2012; Kuhls et al., 2008; Mauricio et al., 2006). Some microsatellite MLGs of this species are sampled in both the Ancient and the New World (Kuhls et al., 2011). The *L. infantum* MLEE MLG 'MON 1' is another typical overrepresented, widespread genotype (Tibayrenc et al., 1990). Near-clades are also observed in the *L. infantum* complex. In Turkey and Cyprus, they show concordance between (1) STRUCTURE, NJ trees and factorial correspondence analysis and (2) between K26-PCR, MLEE and microsatellites (Gouzelou et al., 2012). Near-clading agreement between Internal Transcribed Spacer, MLEE, MLST, miniexon typing and gp63 intergenic RFLP has also been observed (Mauricio et al., 2006). In Turkey, a monophyletic near-clade has been delimitated by WGS and PCR-MLST, with concordance with MLEE (Rogers et al., 2014). In Latin America, near-clading agreement is obtained between: (1) STRUCTURE and NJ and (2) MLEE and microsatellites (Kuhls et al., 2011). Geographical separation apparently interferes with the near-clading pattern observed in the *L. infantum* complex, but can explain it only partially. The clonal MLEE MLG MON 1 exhibits an RD pattern. As a matter of fact, microsatellite analysis reveals the existence of three near-clades within this MLEE MLG, which is itself a tiny subdivision of *L. infantum*. Within-MON 1 near-clades, which are corroborated by STRUCTURE, factorial component analysis and distance methods (congruence principle), are widespread in Brazil and Paraguay. Of a total sample of 173 strains, the ubiquitous microsatellite MLG 10 has been sampled 52 times in 14 Brazilian states and Paraguay (Ferreira et al., 2012). High microsatellite diversity within this group of parasites, which appeared monomorphic with MLEE, illustrates the fact that 'identical genotypes' is a relative notion, that depends highly on the resolution power and mutation rate of the concerned marker (see 'Identical Multilocus Genotypes is a Relative Notion'). The use of powerful markers (WGS and PCR-MLST) has made it possible to discriminate a monophyletic group (near-clade) in Turkish *L. infantum* strains

(Rogers et al., 2014). This near-clade corresponds to a unique MLEE MLG, again another case where a powerful marker (PCR-MLST) reveals additional genetic variability within a given MLG. Contrary to what has been claimed (Ramírez and Llewellyn, 2014, 2015), this case does not challenge the RD model. On the contrary, it is a fine illustration of it. As a matter of fact, within this near-clade, reproduction is 'primarily clonal'; 'intraspecific linkage patterns show low levels of recent recombination', which 'may have been an infrequent event'; and one observes 'mainly clonal reproduction in the parasite population', according to the very terms of the authors (Rogers et al., 2014).

The species *Leishmania killicki* appears to be a phylogenetic subdivision (near-clade) of *Leishmania tropica* (Chaara et al., 2015a,b). For this reason, the authors have proposed to rename it '*L. killicki* syn. *Tropica*'. Within *L. killicki* as well as non-*killicki* strains of *L. tropica*, various genetic subdivisions (RD patterns) are apparent (Chaara et al., 2015a,b). In Asia, the population structure of *L. tropica* has remained stable for 55 years (Schwenkenbecher et al., 2006).

The question of PCE in African trypanosomes responsible for human African trypanosomiasis (HAT) and cattle diseases (the *T. brucei* complex) is not simple. After the seminal article by Tait (1980) and successful mating experiments (Jenni et al., 1986), *T. brucei* has been considered as a sexual species ('panmictic prejudice'). However, we have proposed that it could undergo clonal evolution (Tibayrenc et al., 1990). Near-clading in the *T. brucei* complex is not obvious. *T. brucei* is traditionally composed of three subspecies, namely, *T. brucei gambiense* (West African HAT), *T. brucei rhodesiense* (East African HAT) and *T. brucei brucei* (cattle disease). These three subspecies have different geographical distributions and different pathogenicities, although these specificities are not clear-cut. The three subspecies do not correspond to clear genetic subdivisions that could be equated to near-clades. However, the so-called *T. brucei gambiense* group I (within the subspecies *T. brucei gambiense*) does correspond to a monophyletic near-clade (Balmer et al., 2011; Koffi et al., 2009; Mathieu-Daudé et al., 1994; Weir et al., 2016). The so-called *T. brucei brucei* 'Kiboko B' (Balmer et al., 2011) appears to be a near-clade too. LD is recorded in *T. brucei gambiense* (Morrison et al., 2008b) and *T. brucei rhodesiense* (Duffy et al., 2013). *T. brucei gambiense* I seems to be strictly clonal (Koffi et al., 2009, 2015), a hypothesis that is strongly supported by WGS analysis (Weir et al., 2016).

Near-clades can be observed in *Trypanosoma congolense* (the subgroups Savannah, Forest and Kilifi) (Holzmuller et al., 2010). It has been postulated

that mating was frequent in the Savannah group (Morrison et al., 2009), a proposal that has to be confirmed by more data and has been questioned in 2015 (Koffi et al., 2015). Strong LD is observed within this group (Morrison et al., 2009).

LD and near-clades are present in *T. evansi* (McInnes et al., 2012) and *T. rangeli* (Hamilton et al., 2011). *Trypanosoma vivax*, for which we have hypothesized clonal evolution (Tibayrenc et al., 1991a), exhibits strong LD (Duffy et al., 2009). However, Koffi et al. (2015) consider that the population structure of this species still has to be clarified.

In summary, when parasitic protozoa are considered, the PCE pattern is quite clear in *T. cruzi*, many *Leishmania* species, *G. intestinalis* and *T. gondii*. *P. falciparum* and *P. vivax* do exhibit clear indications of clonal population structure in some populations, but definitely do not fit the typical PCE pattern. Other species call for further studies.

3.2 Fungi and yeasts

According to Taylor (2015), in fungi, clonality, which the author equates to restrained recombination, can have extrinsic and intrinsic cause. Extrensic causes include dispersal (bottleneck/founder effects leading to a deficiency of mating types and adaptation to new hosts) and hybridization between very divergent parental genotypes, leading to the impossibility of meiosis, a mechanism invoked also by Avise (2015) to explain asexuality in clonal vertebrates. Intrinsic causes include mitotic clonality, selfing and intratetrad mating.

In *Candida albicans*, the genetic distances calculated from ca3 markers, MLEE, MLST and microsatellites are highly correlated (McManus and Coleman, 2014). This meets the criteria of our *g* test of LD (Tibayrenc et al., 1990). This species shows a very clear near-clading pattern (Tibayrenc and Ayala, 2012), corroborated by several genetic markers, with strong links with phenotypes (drug resistance, pathogenicity) (Chávez-Galarza et al., 2010; McManus and Coleman, 2014; Tavanti et al., 2005). Worldwide-distributed major clades are subdivided into various minor clades (RD pattern) (McManus and Coleman, 2014; Tavanti et al., 2005). Recombination is rare among and within clades (McManus and Coleman, 2014).

LD and ubiquitous genotypes are observed in *Candida dubliniensis* (Badoc et al., 2002).

We have proposed (Tibayrenc et al., 1991a) that *Cryptococcus neoformans* and its serotype subdivisions could undergo clonal evolution. There is strong LD revealed by amplified fragment length polymorphism (AFLP), MLST,

PCR fingerprint, RAPD, RFLP and gene sequences in the two sibling species *Cryptococcus gattii* and *C. neoformans* (Bovers et al., 2008; Lin and Heitman, 2006; Ngamskulrungroj et al., 2009). In these two species, LD is observed between genetic types and serotypes (Campbell and Carter, 2006). In *C. gattii*, the clonal genotype responsible for the Vancouver epidemics has also been sampled in San Francisco, and is identical to the NIH 444 strain isolated in 1970 (Carriconde et al., 2011; Chaturvedi and Chaturvedi, 2011). It is supposed to have originated from Australia by 'same sex mating' (Fraser et al., 2005). Ubiquitous clones are uncovered in *C. neoformans* var. *grubii*. One MLG has been sampled from 1996 to 2007 in Africa and Asia. Another one has been recorded from 1983 to 2009 in North and South America, in Europe and in Asia (Khayhan et al., 2013). *C. gattii* and *C. neoformans* also exhibit very typical near-clades (Tibayrenc and Ayala, 2014b), corroborated by various molecular markers, including AFLP, MLST, PCR fingerprinting, RAPD and RFLP, and strongly linked to serotypes (Bovers et al., 2008; Campbell et al., 2005; Campbell and Carter, 2006; Carriconde et al., 2011; Chaturvedi and Chaturvedi, 2011; Fraser et al., 2005; Khayhan et al., 2013; Lin and Heitman, 2006; Litvintseva and Mitchell, 2012; Ngamskulrungroj et al., 2009; Voelz et al., 2013; Xu, 2006a). In *C. gattii*, there are 4 'molecular types' (near-clades) I to IV. In molecular type II, there are three clonal groups a, b and c (RD pattern) (Chaturvedi and Chaturvedi, 2011; Ngamskulrungroj et al., 2009; Voelz et al., 2013). In the cluster (near-clade) VGI of this species, four subdivisions (C1−4) are observed (RD pattern) (Campbell et al., 2005).

Fusarium oxysporum exhibits LD between various markers. Near-clades in this species are corroborated by AFLP, multi-gene phylogenies, PFGE, RAPD and RFLP (Fourie et al., 2011).

Lastly, in *Pneumocystis jirovecii*, identical MLGs have been sampled in 10 European hospitals from different countries for 9 years, and in particular patients for 8 weeks (Matos and Esteves, 2010). However, the evidence for PCE in this species still is weaker than for the species listed earlier.

3.3 Bacteria

Three paradigmatic examples will be reviewed for bacteria, namely, *E. coli*, *N. meningitidis* and *Mycobacterium tuberculosis*. Other species will be more briefly treated.

E. coli could be considered as a kind of 'bacterial twin' for eukaryotic pathogens that perfectly fit the PCE model, such as *T. cruzi*, *G. intestinalis*,

T. gondii, *C. albicans* and *C. neoformans*/*C. gattii*. This is so to the point that through a blind lecture, genetic data dealing with *E. coli* and these eukaryotic species could be confounded. In *E. coli*, LD is strong between various markers (MLEE, RAPD, RFLP, MLST, WGS) (Chaudhuri and Henderson, 2012; Tenaillon et al., 2010) and between genetic markers and phenotypes (Miller and Hartl, 1986). This species counts among the most demonstrative cases of near-clading. The MLEE A, B1, B2, D groups identified in the historical ECOR collection of strains by pioneer studies (Ochman and Selander, 1984; Whittam et al., 1983) have been fully corroborated, and their permanency as well, by many studies relying on various genetic markers (Chaudhuri and Henderson, 2012; Clermont et al., 2011; Denamur et al., 2010; Walk et al., 2009; Wirth et al., 2006). The fact that the rate of recombination in *E. coli* almost equals that of mutation (Bobay et al., 2015) has not prevented the long-term stability of a strong structuring into typical near-clades. WGS makes it possible to refine this picture and to uncover new near-clades, especially in environmental strains (Chaudhuri and Henderson, 2012; Luo et al., 2011). Lastly, *E. coli* near-clades seem to exhibit phenotypic differences (Chaudhuri and Henderson, 2012; Miller and Hartl, 1986).

N. meningitidis is described as a paradigmatic case of the 'semiclonal model' (Maiden, 2006) or 'epidemic clonality model' (Maynard Smith et al., 1993), that is, occurrence of occasional bouts of clonality in an otherwise highly recombining species (Fig. 3). However, LD is considerable throughout the range of the species, with 'distinct cocirculating lineages that constitute a small subset of the possible allele combinations' (i.e., LD) (Buckee et al., 2008). LD is confirmed by MLEE, MLST (with loci different from the MLEE loci) and RAPD (Bart et al., 2001). Remarkably, LD includes a strong association between MLST genotypes [sequence types (STs)] on the one hand and phenotypes (capsular serogroups a, d: 'finetypes' {antigen sequence typing}) on the other hand (Joseph et al., 2011; Vogel et al., 2010). We have shown that many data are at odds with the semiclonal model in *N. meningitidis* and have proposed that this bacterium fits better the PCE model (Bart et al., 2001; Tibayrenc and Ayala, 2012, 2015c). *N. meningitidis* shows abundant examples of widespread, persistent MLGs (Bennett et al., 2007; Caugant and Maiden, 2009; Maiden, 2008; Vogel, 2010). This is the case also for *Neisseria gonorroeae* and *Neisseria lactamica* (Bennett et al., 2007). Lastly, *N. meningitidis* exhibits typical near-clades, whose stability in space and time is corroborated by several genetic markers (Bart et al., 2001; Achtman, 2004; Falush, 2009). These near-clades are correlated

with gene content (Joseph et al., 2011) and serotypes (Buckee et al., 2008; Caugant, 2008; Caugant and Maiden, 2009; Joseph et al., 2011). WGS deep phylogeny (Budroni et al., 2011) confirms the stability of the near-clades ('phylogenetic clades') at an evolutionary scale, which challenges the 'semiclonal' model (Maiden, 2006). Contrary to what Ramírez and Llewellyn (2015) state, the phylogenetic clades show a typical RD pattern. As a matter of fact, several subclusters, which correspond to 'clonal complexes' (CCs), are visible within each of them (Fig. 5). These CCs are widespread and persist for many years in spite of recombination (Budroni et al., 2011).

Establishing deep phylogeny was impossible with MLST, even with 20 loci (Didelot et al., 2009b), which shows that this result is due, not to abundant recombination, but rather to an unadapted resolution power of MLST at this level of phylogenetic divergence in *N. meningitidis*. The manifestation of a stronger near-clade pattern (Budroni et al., 2011) by the use of high-resolution markers (WGS) is a clear illustration of the efficacy of the congruence criterion. PCE features are observed in pathogenic strains of *N. meningitidis*. Commensal strains seem to exhibit a different pattern, with more genetic diversity (Caugant, 2008). It is possible that PCE corresponds to a specific evolutionary strategy of the species to adapt to a specific environment and a parasitic life.

In *M. tuberculosis*, considered as highly clonal species (Henriques-Normark et al., 2008), LD has been observed between various markers, including SNPs and large sequence polymorphisms (Comas and Gagneux, 2009), and between MIRU microsatellites and IS 6110 markers (Supply et al., 2003) (*g* test; Tibayrenc et al., 1990). The W strain has spread from the United States to France; and the Beijing family strains have a global distribution (Bifani et al., 2002). This species shows a clear near-clading pattern with 6 'phylogenetic groupings' with differences in their geographical distribution, host specificity (ethnicity) and pathogenicity (Achtman, 2008). It is remarkable that this near-clading pattern was not apparent with less discriminating markers, which seemed to show that this species was almost monomorphic. The use of more powerful markers (SNPs, WGS) has revealed these hidden subdivisions (Achtman, 2008; Comas and Gagneux, 2009; Smith, 2012). It is relevant to note that the phylogeny based on the '3R genes' (DNA repair, recombination, replication), which are under intense selective pressure, parallels the overall *M. tuberculosis* phylogeny (dos Vultos et al., 2008).

Other cases of bacterial models are reviewed below.

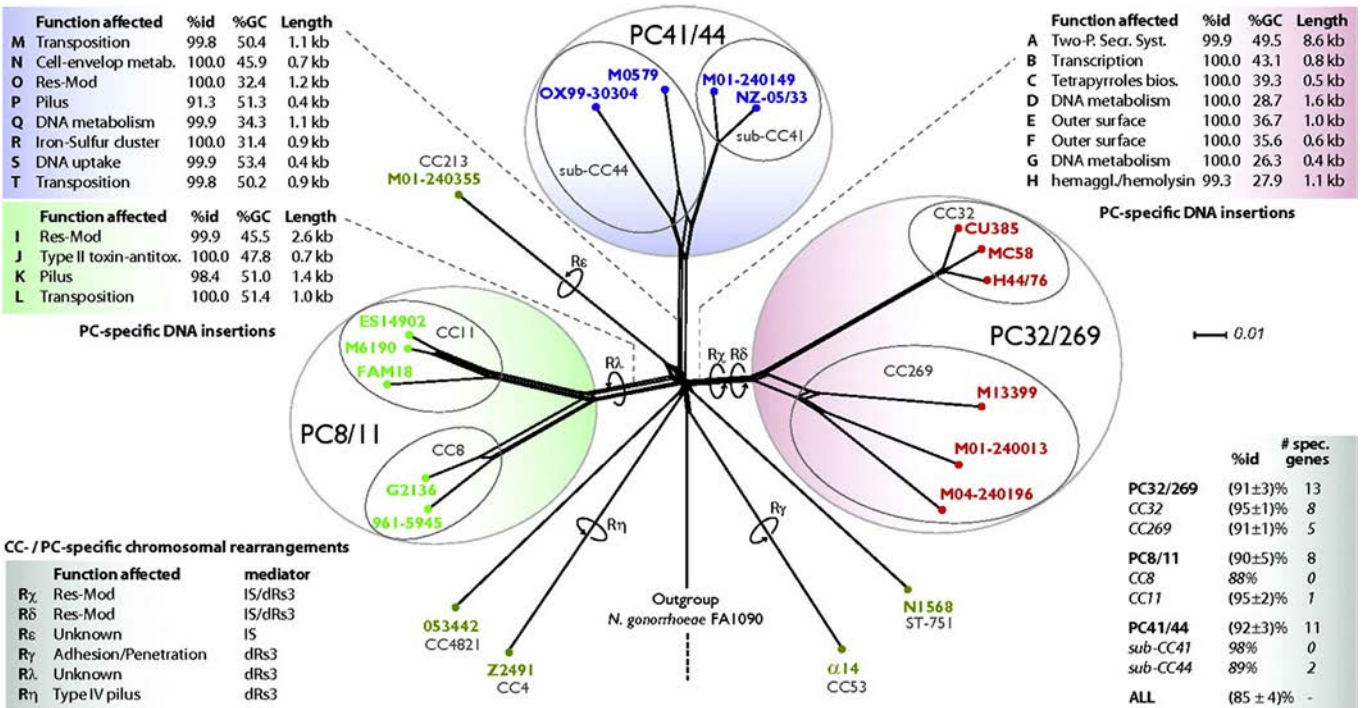

Figure 5 Deep phylogenies and Russian doll patterns in *Neisseria meningitides*. Within the 'phylogenetic clades' (near-clades), additional subdivisions are clearly visible. Each phylogenetic clade is composed of several clonal complexes that are stable in space and time ('Russian Doll pattern'). *After Budroni, S., Siena, E., Dunning Hotopp, J.C., Seib, K.L., Serruto, D., Nofroni, C., Comanducci, M., Riley, D.R., Daugherty, S.C., Angiuoli, S.V., Covacci, A., Pizza, M., Rappuoli, R.E. Moxon, E.R., Tettelin, H., Medini, D., 2011.* Neisseria meningitidis *is structured in clades associated with restriction modification systems that modulate homologous recombination. Proc. Natl. Acad. Sci. U.S.A. 108, 4494–4499.*

The *Bacillus cereus* group is subdivided into 3 'clades' (near-clades) that are corroborated by both unweighted pair group method with arithmetic mean (UPGMA) analysis and the ClonalFrame software. The near-clades exhibit a pathogenic specificity, since all strains of *Bacillus anthracis* are in clade 1, whereas clade 3 shows no pathogenic genotypes (Didelot et al., 2009a).

B. anthracis, which is a subdivision of *B. cereus*, is itself divided into near-clades, although its overall genetic diversity is very limited, to the point that it has been considered a monoclonal species (Achtman, 2004). However, the use of markers that have an adequate resolution make it possible to unravel the presence of 12 stable, ubiquitous subgroups (near-clades) (Kenefic et al., 2010). This presence of near-clades within a near-clade is a typical RD pattern.

Bartonella bacilliformis exhibits strong LD, corroborated by MLST, AFLP and Infrequent Restriction Site PCR (Chaloner et al., 2011). In this species, the MLG ST 1 has been sampled repeatedly at various places in Peru from 1960 to 2007. ST8 has been sampled twice 150 km and 9 years apart, in spite of an inferred 'strong influence of recombination' (Chaloner et al., 2011).

Bartonella henselae has strong LD (Mietze et al., 2011). This species shows three major clusters (near-clades) corroborated by six different genetic markers (Mietze et al., 2011).

In *Bartonella quintana*, the same MLG has been sampled over 60 years and three continents (Arvand et al., 2010).

Borrelia burgdorferi in North America is subdivided into discrete clusters (near-clades) revealed by phylogeography (Kurtenbach et al., 2010).

Burckholderia pseudomallei, in spite of a supposedly high recombination rate, exhibits a strong phylogenetic signal and reveals a deep phylogeny by the use of WGS/SNP, 'as individual lateral gene transfer events do not involve a large enough portion of the genome to disrupt the core phylogenetic patterns'. It is interesting to note that MLST was inappropriate to reveal this deep phylogeny (Pearson et al., 2009a).

Campylobacter coli is subdivided into three clearly differentiated clades (near-clades) (Sheppard et al., 2010).

The *Enterococcus faecium* hospital cluster represents a 'genogroup' (near-clade) corroborated by MLST, AFLP and variability of the dispensable genome (Willems, 2010; Willems et al., 2011).

In *Legionella pneumophila*, the clonal strain 'Paris' has a worldwide distribution (Gomez-Valero et al., 2009). *L. pneumophila* exhibits also strong LD (Edwards et al., 2008). MLST analysis reveals the presence of 'sequence clusters' (near-clades) in this species (Edwards et al., 2008).

Listeria monocytogenes exhibits LD and near-clades, corroborated by both MLST and ribotyping (den Bakker et al., 2008, 2010).

Mycobacterium bovis exhibits LD and near-clades corroborated by various markers: deletions, variable numbers tandem repeats (VNTRs), spoligotyping (Smith, 2012).

Pseudomonas aeruginosa is another species considered as frequently recombining (Pirnay et al., 2009). However, LD is strong: (1) between different markers: PFGE with microsatellites, multiple locus VNTR and MLST (van Mansfeld et al., 2010) and (2) within the core and accessory genomes and between them (Wiehlmann et al., 2007). *P. aeruginosa* shows examples of worldwide clones (Pirnay et al., 2009; van Mansfeld et al., 2010; Wiehlmann et al., 2007). Moreover, clear indications for near-clading are confirmed by several genetic markers (van Mansfeld et al., 2010). Near-clades are also delimited by microarrays relying on 58 markers (Wiehlmann et al., 2007) and by strongly selected genes (Pirnay et al., 2009). However, WGS deep phylogenies are not available.

In *Pseudomonas syringae*, the same MLG has been sampled in the United States in 1965 and in Japan in 1979 (Sarkar and Guttman, 2004). *P. syringae* also shows LD, and clear near-clades, although they have been identified only by MLST (Sarkar and Guttman, 2004).

S. enterica is subdivided into five lineages (near-clades) corroborated by both ClonalFrame and STRUCTURE, as evidenced by sequencing 10% of the core genome. These near-clades are highly linked to the serovars, and to pathogenicity (Didelot et al., 2011).

In *S. typhi*, WGS shows that the H58 clade (near-clade) is widespread worldwide. It exhibits a clear microclustering (RD pattern), although the most recent common ancestor is estimated to be dated to the year 1959 only (Wong et al., 2015). This shows that 'measurably evolving pathogens' (Biek et al., 2015), that is, pathogens whose epidemiology can be followed up through WGS and time-serial samplings, are not only limited to fast-evolving viruses but also concern bacteria (Wong et al., 2015) and even parasitic protozoa (Imamura et al., 2016).

Staphylococcus aureus shows strong LD (Smyth and Robinson, 2010). This species' MLEE MLGs often have intercontinental distributions (Musser et al., 1990). *S. aureus* exhibits two near-clades. This near-clading pattern uncovered by modern markers (Feil et al., 2003; Smyth and Robinson, 2010) parallels the one revealed by MLEE 25 years ago (Musser et al., 1990).

The 'highly recombining' species, *S. pneumoniae* (Pérez Losada et al., 2006) exhibits LD between MLST (core genome), microarrays and

accessory genes (Dagerhamn et al., 2008; Henriques-Normark et al., 2008). This species has been analysed by WGS and SNPs. Again, these highly resolutive markers reveal the deep phylogenies of clear near-clades with significant correlation between core and accessory genome phylogenies, and between phylogenies and antibiotic resistance (Chewapreecha et al., 2014; Croucher et al., 2011; Muzzi and Donati, 2011). WGS/SNP analysis reveals the presence of 33 primary clusters subdivided into 183 secondary clusters (RD pattern) (Chewapreecha et al., 2014).

Streptococcus pyogenes also shows clear indications of near-clading, corroborated by different markers and WGS. Ten 'major subclones' are each composed of various CCs (RD pattern) (Beres et al., 2010).

In *Vibrio cholerae*, *Vibrio parahaemolyticus* and *Vibrio vulnificus*, successful clonal lineages persist for decades (Bisharat, 2010). *V. vulnificus*, presented as a 'model of clonal evolution', exhibits 2 clusters (near-clades) corroborated by both MLEE and MLST. One contains mainly environmental strains, whereas the other one contains mainly clinical strains. The second cluster is itself clustered (RD pattern) (Bisharat, 2010).

In *Xanthomonas campestris*, some MLGs pertaining to the same 'pathovar' have persisted worldwide from 1949 to 1981(Fargier et al., 2011). *X. campestris* exhibits several clusters corroborated by both NJ tree and Splitstree, which could correspond to near-clades, although the evidence is based on only one marker (MLST). The clusters correspond to the 'pathovars' (Fargier et al., 2011).

In summary: *E. coli*, *M. tuberculosis*, *S. aureus* and *S. typhi* perfectly fit the PCE model. This seems to be also the case for *N. meningitidis*, which is unexpected, since this species was considered as highly recombining. Other species show clear indications of PCE patterns. However, they deserve more detailed analysis.

3.4 Viruses

Many viral species are recognized as being mainly clonal (with low rates of recombination) (Holmes, 2009, 2013). It has been proposed that clonality (defined as restrained recombination) is the standard evolutionary mode for viruses because recombination is 'largely inconsequential' (Perales et al., 2015). As a consequence, viral lineages (near-clades) can be defined (Perales et al., 2015), as we have proposed it for viruses as well as for other pathogens (Tibayrenc and Ayala, 2012). Recombination rate seems to (1) vary between virus categories and (2) be rather specific of a given category (Pérez-Losada et al., 2015).

Many virus species do show structures similar to near-clades and RD. Of course, the rapid turnover of viral genomes entails that the oldness of these viral near-clades cannot be compared with that of other microbes' near-clades. Still the fact remains that they show discreteness, as well as permanency in space and time, including in some highly recombining species.

Many cases are illustrative of a PCE pattern in viral species. Maybe the most demonstrative one is the dengue virus (DENV). However, it is difficult to hierarchize the level of evidence in the many species cited below.

DENV shows four phylogenetic subtypes (DENV 1–4: near-clades) that are linked to serotypes (Holmes, 2008; Weaver and Vasilakis, 2009). It has been inferred that the linkage between subtypes and serotypes was less strict than what was classically thought. However, it remains statistically very strong (Katzelnick et al., 2015). The four subtypes are stable since at least 1943, and circulate sympatrically, especially in Latin America and Asia, which provides ample opportunity for mating (Messina et al., 2014). The phylogenetic subtypes are subdivided into 'genotypes' (Weaver and Vasilakis, 2009) or 'clades' (Raghwani et al., 2011) (RD pattern).

The highly recombining Human Immunodeficiency virus I (HIV I) exhibits stable groups M, N, O and P linked to phenotypes (epidemiology, transmissibility, infectivity for different cellular types), in spite of the existence of circulating recombinant forms (Etienne et al., 2011; Lam et al., 2010; Ndung'u and Weiss, 2012). Within the M group nine subgroups can be discriminated (Pérez-Losada et al., 2015) (RD pattern).

Chikungunya virus exhibits three major widespread 'phylogroups' I, II and III (near-clades), which are clearly linked to geographical separation (Cui et al., 2011). However, within the main phylogroups, some genotypes are widespread and stable in time. The phylogroup II has been identified in India, Indonesia, Malaysia, the Philippines, Thailand and the United States. The phylogroup III is subdivided into 'subgroups' III a, b and c (RD pattern). The 'subgroup' IIIa includes strains isolated from 1952 to 1986 in Tanzania, Senegal, Congo and India. The 'subgroup' IIIc has been discovered in Bangladesh, California, China, India, Italy, Malaysia, Singapore, Sri Lanka and Thailand. Recent importations can account for some of these results dealing with ubiquitous genotypes. However, it would be tentative to explain this overall pattern by only a trivial Wahlund effect.

The Ebola virus shows three 'groups' A, B and R (near-clades), with some indices of recombination (Wittmann et al., 2007).

The Hepatitis A virus (HAV) exhibits three main 'genotypes' I, II and III with a worldwide distribution (Vaughan et al., 2014). Within these main

'genotypes' (near-clades), various subtypes (IA, IB, IIA, IIB, IIIA, IIIB) can be identified and occur sympatrically (RD pattern). There are three major clusters within the subgroup IA (RD pattern).

The Hepatitis B virus has eight genotypes A to H and several subgenotypes A1−6, B1−8, C1−7, D1−7, and F1−4 (RD pattern). One observes specificities for pathogenicity and geographical distribution. Recombinant forms are recorded (Araujo et al., 2011).

The Hepatitis C virus has six to seven main genotypes divided into up to 67 'subtypes' (near-clade + RD pattern) (Fishman and Branch, 2009; Jackowiak et al., 2014; Morel et al., 2011; Simmonds et al., 2005).

The Hepatitis E virus has four 'genotypes' and many 'subtypes' (near-clade + RD pattern) (Purdy and Khudyakov, 2011).

The measles virus is subdivided into several 'clades' or 'genotypes' (WHO, 2003; Mankertz et al., 2011). However, these data have not been analysed in terms of multilocus population genetics and phylogenetics. This should be done before these 'clades/genotypes' can be equated to near-clades.

The rabies virus (RABV) exhibits two distinct lineages in West Africa that shows partial allopatry, but can be found sympatrically (Hayman et al., 2011). At the level of the whole species, three 'clades' (near-clades) can be identified (I, South East Asia; II, worldwide; III, only bats and raccoons in North and South America). Two of these clades are present in China (Zhang et al., 2009). Various subdivisions can be observed within these clades (Zhang et al., 2009) (RD pattern). Intra- and interclade recombination events can be detected. However, their frequency seems to be very low (Liu et al., 2011).

The varicella-zoster virus according to a new common nomenclature, has five major 'clades', partially linked to geography, and several subclades (near-clades + RD pattern) (Schmidt-Chanasit and Sauerbrei, 2011).

The West Nile virus has two major 'lineages' (near-clades): I and II, and four others (III to VI), the existence of the latter being questionable. Lineages seem to be associated with specific pathogenic properties (Pesko and Ebel, 2012; Zehender et al., 2011). Lineage I Is subdivided into 1a, 1b and 1c. The sublineage 1a is itself subdivided into A and B (Zehender et al., 2011) (RD pattern). These different subdivisions have specific geographical distributions. They are not explainable by a Wahlund effect only. Several of them are widespread.

The numerous examples cited earlier show that in many micropathogen species, not only parasitic protozoa but also fungi, bacteria and

viruses, the marks of PCE are clear. This manifests that these pathogens, although phylogenetically quite diverse, share this important common evolutionary trait. This common pattern is so strong in some cases that blindly read genetic data from *T. cruzi*, *G. intestinalis*, *T. gondii, C. albicans*, *C. neoformans/C. gattii*, *E. coli*, as examples, could make it difficult to identify which case is under survey. The existence of such a common evolutionary trait has nothing unlikely and probably is a mark of the adaptation to parasitism that is shared by all these pathogens. Another important evolutionary trait, namely, reduction of genome size, is common to many organisms having a parasitic lifestyle (Buscaglia et al., 2015). Of course, the level of evidence is not the same among these species. This is due to the fact that progress among the different fields of research concerned is not the same from one species to another. Moreover, evolutionary specificities of the different pathogens condition this progress. For example, the tiny size of viral genomes makes them much more accessible to routine WGS. Also, technical specificities bring strong limitations to evolutionary analyses. As examples, *Plasmodium* parasites are much more difficult to culture than trypanosomes and *Leishmania*, and *Pneumocystis* cannot be cultivated. Lastly, although many species appear to undergo PCE, this is not the case for *P. falciparum* and *P. vivax* for example. These parasites, although they are capable of clonal propagation, do not meet the criteria of the PCE model (near-clades that are stable in space and time, as a result of the clonality threshold).

We will now expose more in detail some relevant aspects of the PCE model.

4. THE 'STARVING SEX' HYPOTHESIS

Clonality in *P. falciparum* and *P. vivax* at first view clashes with the classical notion of an obligatory sexual cycle in these parasites. Clonality in these species is generally explained by the fact that MCIs are rare in low transmission areas. Thus, mating between partners having different genotypes (outcrossing) is impossible. The parasite therefore undergoes selfing, which leads to clonality (Anderson et al., 2000). Many authors explain clonality in *Plasmodium* by this hypothesis (see, for example, Branch et al., 2011; Conway, 2007; Falk et al., 2015; Iwagami et al., 2012; Miotto et al., 2013; Neafsey et al., 2008; Weedall and Hall, 2014). We have called this situation the 'starving sex hypothesis', or 'passive clonality' (Tibayrenc

and Ayala, 2014a). We have shown that in both *P. falciparum* and *P. vivax*, a large amount of data are at odds with starving, which suggests that these parasites may also undergo clonality by in-built mechanisms. We have presented many examples of such cases (Tibayrenc and Ayala, 2014a). Some of them will be cited now. A highly significant LD is observed in *P. falciparum* populations of Papua New Guinea and Zimbabwe, although transmission is strong in New Guinea and MCIs are frequent in Zimbabwe (Anderson et al., 2000). LD is stronger in Zimbabwe than in Brazil, even though transmission is higher in Zimbabwe (Anderson et al., 2000). Clonality has been evidenced in *P. falciparum* despite high transmission in Kenya and Cameroon (Annan et al., 2007; Razakandrainibe et al., 2005). WGS and typing by 86,158 SNPs has shown that inbreeding was very strong in Papua New Guinea in spite of high transmission (Manske et al., 2012). Inbreeding has been shown in *P. vivax*, in spite of frequent MICs, a result considered 'puzzling' by the authors (Ferreira et al., 2007).

Starving sex probably occurs in *Plasmodium* and in other microparasites. However, the many examples we have cited (see also Tibayrenc and Ayala, 2014a) lead to not rule out the hypothesis of in-built clonality, the more so because the two hypotheses are not mutually exclusive. We have proposed to move the debate in *Plasmodium* from sexuality versus clonality to starving sex versus in-built clonality (Tibayrenc and Ayala, 2014a). If it is verified that the starving sex hypothesis is unsatisfactory in *P. falciparum*, an important epidemiological implication will be that LD cannot be used as a reliable indicator of the efficacy of control measures (Volkmann et al., 2012b), since clonality and transmission rate will not be correlated.

Starving sex by scarcity of MCIs has been proposed for bacteria: it would lead to selfing and 'invisible sex' (Balloux, 2010). However, there is no experimental evidence for it, and it can be suspected that MCIs in bacteria are grossly underestimated. In viruses, lack of recombinants in HAV has been explained by starving sex (Vaughan et al., 2014). However, MCIs are recorded for this virus in Korea and Japan. Starving sex has been inferred to explain higher recombination rates of HIV-1 in Africa, where MCIs are more frequent (Pérez-Losada et al., 2015). Superinfection exclusion could favour starving sex in viruses (Jackowiak et al., 2014; Perales et al., 2015).

Starving sex actually amounts to a Wahlund effect, by physical separation of putative recombinant partners. We propose to extend it to all cases where recombination is not restrained by in-built properties of the organisms, but rather, by lack of physical opportunity for mating.

5. A DEBATE IN THE DEBATE: UNISEX/SELFING/INBREEDING VERSUS 'STRICT' CLONALITY

Our definition of PCE clearly states that it is defined by strongly restrained recombination only. This concept definitely includes selfing/strong inbreeding and different forms of parthenogenesis as well as mitotic propagation (Tibayrenc and Ayala, 1991, 2002, 2012). As we have recalled it, this definition is shared by many authors working on micropathogen population genetics and general evolution (Table 2). However, some authors prefer to distinguish between 'strict' clonality (i.e., mitotic propagation) and other cases where recombination is restricted too. This is the case for 'unisexual reproduction' (Feretzaki and Heitman, 2013), selfing and inbreeding (Bobay et al., 2015; Rougeron et al., 2009, 2010). This is a matter of definition.

It is informative to refine our views on these evolutionary phenomena. However, we have insisted on the fact that in our view, the most relevant common trait is restrained recombination, since it appears to be the major adaptive strategy that pathogens use to escape the recombinational load (Agrawal, 2006; Becks and Agrawal, 2012), to spread successfully well-adapted MLGs, and therefore to adapt to a parasitic lifestyle. Mitotic propagation and all kinds of unisex/selfing/inbreeding are the various means used by pathogens towards reaching this goal. Selfing/inbreeding is certainly very frequent in bacteria (Maiden, 2008; Caugant and Maiden, 2009) and viruses (Jackowiak et al., 2014; Perales et al., 2015). However, in general, the authors do not distinguish it from clonality. Nevertheless, Bobay et al. (2015) consider that selfing in bacteria should be distinguished from mitotic clonality even if it do not make a difference on the apparent structure of the population. With respect to applied research, restrained recombination is the most important parameter to consider for strain typing (molecular epidemiology) and tracing of genes of interest.

When haploid pathogens are considered, it is impossible to distinguish selfing/inbreeding from mitotic clonality by population genetic tests. The means used for eukaryotic microorganisms rely on tests based on the hypothesis of diploidy (de Meeûs et al., 2007a). It is asserted that mitotic clonality should generate an excess of heterozygotes, till fixed heterozygosity, whereas selfing/inbreeding should produce the opposite. This very statement is disputable even if the hypothesis of diploidy is retained. In the strictly clonal cladocera *Daphnia pulex*, long-term experiments have shown that the tendency is actually loss of heterozygosity through mitotic

recombination, which is 1000 times more frequent than increasing heterozygosity by accumulation of divergent mutations (Omilian et al., 2006). In *Leishmania mexicana* and *L. braziliensis* (Rogers et al., 2011) and *T. cruzi* (Yeo et al., 2011), microsatellites show a deficit of heterozygotes, whereas SNP polymorphism shows the contrary, although the markers should show convergent results if selfing was verified. In *G. intestinalis*, heterozygote excess has been considered as evidence for a recent sexual event/hybridization, whereas heterozygote deficit has been rather regarded as an indication of ancient clonal evolution by purifying selection/gene conversion (Andersson, 2012). Caution is needed for the interpretation of heterozygosity statistics of parasite microsatellite data, since strong evidence for LD is found with both positive and negative values for *F*is (Ramírez et al., 2012). Genome-wide mitotic gene conversion rather than selfing has been considered a parsimonious explanation for heterozygote deficit in *T. cruzi* (Llewellyn et al., 2009a). In *T. brucei gambiense* I, which is considered strictly clonal (Weir et al., 2016), long runs of homozygosity have been accounted for by gene conversion and mitotic recombination rather than by selfing.

Even more troublesome, the hypothesis of diploidy has been challenged by many studies, suggesting that parasites and fungi may undergo widespread aneuploidy. The results are especially convincing for *Leishmania* (Boité et al., 2012; Downing et al., 2011; Inbar et al., 2013; Lachaud et al., 2014; Mannaert et al., 2012; Rogers et al., 2011, 2014; Sterkers et al., 2011, 2012, 2014), but are also available for *T. cruzi* (Buscaglia et al., 2015; Minning et al., 2011; Reis-Cunha et al., 2015; Souza et al., 2011). Aneuploidy is also frequent in *C. albicans* and *C. neoformans* (Ene and Bennet, 2014; McManus and Coleman, 2014; Ni et al., 2013). Microsatellite data could seem to clash with these results, since they do not suggest aneuploidy (Rougeron et al., 2015). However, 'the conclusion that microsatellite data support diploidy rather than aneuploidy is not justified. Indeed, PCR amplification of a given locus reveals either one or two bands that correspond to a phenotype and not a genotype. This, for example when applied to microsatellites, does not allow determining the number of allele (hence chromosome copies) present in the genome.' 'Over-replication of one homologue generates two identical alleles and those will be undistinguishable by PCR amplification.' (Lachaud et al., 2014). If widespread aneuploidy occurs, this renders population genetics tests based on the hypothesis of diploidy invalid (Reis-Cunha et al., 2015; Tibayrenc and Ayala, 2012, 2013). Moreover, aneuploidy leads to rapid elimination of heterozygosity

by frequent passage through haploidy (Sterkers et al., 2012). It could therefore be an explanation for the apparent heterozygote deficit frequently observed in *Leishmania* and *T. cruzi*. It has been proposed that aneuploidy in *Leishmania* could be transitory (Rougeron et al., 2015). This is not supported by genomic analyses that deals with natural isolates and not experimental populations (Downing et al., 2011; Imamura et al., 2016). Moreover, even if aneuploidy were transitory, heterozygosity purging at each haploid cycle (Sterkers et al., 2012) should remain.

Lastly, widespread aneuploidy should render impossible any Mendelian mechanism, including meiosis (Lachaud et al., 2014) and hence, endogamy/self-fertilization through meiosis. On the contrary, PCE is perfectly compatible with widespread aneuploidy, and even more, aneuploidy suggests that asexual reproduction (Reis-Cunha et al., 2015) and PCE occur.

We do not argue that population genetic tests relying on the hypothesis of diploidy should be definitely discarded. However, we recommend to use them very cautiously. We have long-privileged LD analysis, which can be performed whatever be the ploidy of the species under study.

6. HOW CAN CLONES SURVIVE WITHOUT RECOMBINATION?

A classical view is that clonal organisms are an evolutionary dead end, due to the so-called 'Muller's ratchet' (inability to purge deleterious mutations through recombination). For this reason, ancient clonal organisms such as bdelloid rotifers have been called 'ancient asexual scandals' (Birky, 2010).

Traditionally, natural selection is considered to be favourable, for it leads to the rapid generation of new MLGs able to face evolutionary challenges (Weismann, 1889). When micropathogens are concerned, this view is widely accepted (Campbell and Carter, 2006; Monis et al., 2009; Prasad Narra and Ochman, 2006 as examples). However, this conventional view has been considered 'glibly' (Charlesworth, 2006). Recombination in viruses has been interpreted as a by-product of mechanical constraints in genome structure (Holmes, 2013), not as an opportunity to generate new MLGs. Other authors consider that the first role of recombination is DNA repair (de Meeûs and Prugnolle, 2011). According to Gorelick and Heng (2010), recombination's most important functions are (1) DNA repair, (2) epigenetic reset at each meiosis, and (3) maintenance of species integrity and ploidy. Moreover, within the concept of recombinational load

(Agrarwal, 2006), the generation of new MLGs is not considered to be always favourable, and could be rather detrimental, especially for species that undergo parasitism. It would be therefore simplistic to consider restrained recombination as a selective drawback.

This is all the more true because, as we have already noted, organisms that are 100% clonal are probably quite exceptional. *T. brucei gambiense* I would be one exception (Weir et al., 2016). There is most times some recombination/hybridization going on (Tibayrenc et al., 1990; Tibayrenc and Ayala, 2012). Occasional bouts of recombination make it possible to benefit from virtually all the advantages of regular recombination (Birky, 2010; Schurko and Logsdon, 2008). In the case of sexual parasitism in vertebrates (gynogenesis, hybridogenesis), although populations are mainly clonal (Avise, 2015), occasional introgression ('genetic leakage') occurs (Lehtonen et al., 2013).

Moreover, organisms that restrain recombination are able to repair their DNA through selfing.

Another possible way to escape Muller's ratchet is by the population size of micropathogens, which is always considerable (Balloux, 2010; Perales et al., 2015).

Lastly, even in the framework of PCE, clonal organisms are able to generate some genetic variability. At the same time, they avoid the recombinational load, Muller's ratchet, the costs for sex and search for partners (Ni et al., 2013).

Widespread aneuploidy is one of the major means for generating variability without recombination between different genotypes. It permits rapid adaptation through gene dose effect, and modulates gene expression (Reis-Cunha et al., 2015). Aneuploidy is considered favourable for clonal populations (cancer, microparasites) (Mannaert et al., 2012). It allows the organisms concerned to explore a large phenotypic landscape, and is associated to drug resistance in *C. albicans* and *C. neoformans*. In a typical yeast population, aneuploidy makes up a large proportion of the genetic variants (Chen et al., 2012). 'Same sex mating' (considered by us to be included in PCE) promotes adaptation through aneuploidy in *C. albicans* and *C. neoformans* (Ene and Bennett, 2014).

Gene conversion and mitotic recombination are other means to generate additional genetic variability within clones (Buscaglia et al., 2015; Calo et al., 2013; Flot et al., 2013; Omilian et al., 2006).

Ackermann (2015) has proposed that phenotypic heterogeneity in genetically identical microorganisms was maintained through (1) stochastic gene expression, (2) periodic oscillations in cellular functions, (3) differential

cellular ageing, (4) cellular interactions by metabolites or physical contacts, and (5) epigenetics.

For the reasons exposed above, PCE should definitely not be considered an evolutionary dead end. It appears to be a sustainable, long-term evolutionary strategy that parasitic protozoa, fungi, bacteria and viruses have used to adapt to the specific features of parasitic life. On the contrary, in vertebrates, clonality seems to be a short-term evolutionary dead end (Avise, 2015).

7. MEIOSIS GENES AND EXPERIMENTAL EVOLUTION: WHAT DO THEY TELL US ABOUT PREDOMINANT CLONAL EVOLUTION?

Genes homologous to meiosis genes [homologues of meiosis-specific genes (HMGs); Poxleitner et al., 2008) are frequently observed in micropathogens. The 'meiosis toolkit' is a set of eight genes present in animals, plants, fungi, and protists. If they are all present in a given organism, it would be an indication that meiosis occurs (Schurko and Logsdon, 2008). However, the 'meiosis' genes may have other functions in addition to meiosis: 'Evolution is constantly re-using old genes for new purposes' (Birky, 2009). Meiosis genes could serve for DNA repair or mitotic recombination (Birky, 2010) or diplomixis in *Giardia* (Poxleitner et al., 2008). They could also code for unusual, primitive forms of meiosis (Birky, 2005). At best, the meiosis toolkit could be an indication that meiosis occurs, but it says nothing about its frequency. As a matter of fact, it is remarkable that meiosis genes have been identified in organisms that exhibit a typical PCE pattern. They are present in *Giardia* (Birky, 2005; Cacciò and Sprong, 2010; Heitman, 2006; Lasek-Nesselquist et al., 2009; Ortega-Pierres et al., 2009; Monis et al., 2009; Schurko et al., 2008), *Leishmania major* (Birky, 2005; Heitman, 2006), *L. donovani* (Birky, 2005), *T. cruzi* (Heitman, 2006) and *T. vivax* (Duffy et al., 2009), among others. In *Giardia*, there is no apparent correlation between the presence or absence of meiotic genes and the observation of meiotic life cycles (Andersson, 2012). HMGs are present in *Aspergillus fumigatus*, *C. albicans* and the *C. neoformans* complex. However, these fungi 'retain their sexuality' to not disrupt fit MLGs (Ene and Bennett, 2014; Heitman, 2006). All meiosis genes are present in *Penicillium marneffei*, although this fungus is highly clonal (Henk et al., 2012). It is remarkable that meiosis genes are expressed in *T. brucei gambiense* I, which is considered strictly clonal (Weir et al., 2016). Besides, in *Drosophila melanogaster*, seven major meiosis genes are absent (Weedall and Hall, 2014). In *D. pulex*,

meiosis genes are activated even in parthenogenetic lines (Schurko et al., 2009). The authors propose that this cladoceran crustacean has a double machinery parthenogenesis/sexuality. We have also proposed that micropathogens could have a 'sexuality/machinery kit' that would allow them to restrain their recombination to face certain evolutionary challenges (Tibayrenc and Ayala, 2012).

In conclusion, the widespread presence of HMGs is perfectly compatible with PCE and should not be used as evidence against it.

The same is true for experimental genetic exchange in pathogens. Successful laboratory mating has been obtained for *L. major* (Akopyants et al., 2009), *T. brucei* (Jenni et al., 1986) and *T. cruzi* (Gaunt et al., 2003). These experiments show only that the potentiality for genetic exchange exists in these parasites. They say nothing about the frequency of these genetic exchanges and their impact on population structure. They should therefore not be taken as evidence against PCE.

8. IS PREDOMINANT CLONAL EVOLUTION AN ANCESTRAL OR CONVERGENT CHARACTER?

PCE appears to be a common character of many pathogens, a very specific evolutionary strategy used by these organisms, probably to adapt to parasitism. Is it ancestral, or the result of convergence? We have proposed that PCE is commanded by a 'sexuality/machinery kit', acting like a biallelic system. Could such a system have an ancestral origin?

Several indices suggest that the mechanisms involved in recombination (and hence, possibly, also in restrained recombination) are very ancient, with maybe a direct filiation from prokaryotes to eukaryotes. Although clonality is widespread in viruses, most of them retain an active recombination machinery (Perales et al., 2015). Many of the enzymes involved in prokaryotic recombination are homologous to those of eukaryotes (Charlesworth, 2006). In *Giardia*, the meiosis machinery is similar to that of higher eukaryotes, and homologous to bacterial genes involved in DNA repair (Michod et al., 2008).

The 'pre-LUCA (last universal common ancestor)' hypothesis (Holmes, 2013) proposes that many characters of cellular organisms may have been inherited from ancient viruses. The viral eukaryogenesis hypothesis (Holmes, 2013) proposes that ancient viruses may be at the origin of some traits of cellular organisms. The eukaryotic nucleus could originate from the viral envelope. There is still a 'continuous rain of viral genes

into cellular genomes', which could be at the origin of many properties of cellular organisms (Forterre, 2006).

To test the hypothesis of a sexuality/clonality machinery and of its possible ancient origin, it would be necessary to identify and characterize it in organisms that undergo PCE with occasional bouts of recombination/hybridization. *T. cruzi* would be an adequate model for it. If this sexual/clonal kit is characterized, it will be possible to look for homologous genes in bacteria and other micropathogens.

9. CAN PREDOMINANT CLONAL EVOLUTION FEATURES BE EXPLAINED BY NATURAL SELECTION? IN-BUILT MECHANISMS FAVOURING CLONALITY

Many authors defend the view that apparent clonality in micropathogens is mainly explained by natural selection, which would eliminate most possible genetic variants, thus leading to LD and clustering (near-clading). This has been asserted for *N. meningitidis* (Buckee et al., 2008; Caugant, 2008; Caugant and Maiden, 2008; Maiden, 2008). 'Meningococci display signs of a highly recombinogenic population with purifying events and consecutive clonal expansion of fit variants. Their population structure has therefore been categorized as "epidemic"' (Maynard Smith et al., 1993). In viruses, selection and non-selective epidemiological processes have been inferred to account for RNA viruses' population structure (Grenfell et al., 2004). Purging selection is considered a main factor driving HAV population structure (Vaughan et al., 2014). Similarly, natural selection has been inferred as a major parameter for *Toxoplasma* population structure (Su et al., 2003).

The PCE model proposed by us (Tibayrenc and Ayala, 2002, 2012) considers that natural selection (downstream elimination of recombinants) alone cannot account for pathogen population structure. We have rather defended the concept of an upstream inhibition of recombination by in-built properties of the organisms under study. Concerning *N. meningitidis*, it is hard to imagine that natural selection would be the main explanation for stable ubiquitous clonal genotypes and near-clades, which face many diverse ecological environments. Moreover, the existence of deep phylogenies revealed by WGS (Budroni et al., 2011) is hardly compatible with the natural selection model, since it shows that *N. meningitidis* near-clades are stable at an evolutionary scale.

A main challenge to the natural selection hypothesis is that it would amount to eliminating in each generation most possible MLGs to maintain

LD and near-clading, which amounts to a considerable genetic load (Tibayrenc, 1995). This view is shared by scientists working on *Toxoplasma* (Lehmann et al., 2004) and *C. dubliniensis* (Badoc et al., 2002). The natural selection hypothesis in *N. meningitidis* has been challenged by Didelot et al. (2009b). The long-term maintenance of *N. meningitidis* 'lineages' is considered to be compatible with neutral evolution (Budroni et al., 2011).

Natural selection, both positive and purging, certainly has a strong impact on micropathogens' population structure. However, we propose that it is not the main factor responsible for PCE patterns, which are better explained by in-built properties of microbes.

Several in-built traits restraining recombination have been identified in bacteria. In many species, the probability of genetic exchange decreases exponentially with the genetic distance between donor and recipient due to the DNA mismatch repair system (Didelot et al., 2011). This is one of the reasons inferred to explain that, in *S. enterica*, recombination within clades (near-clades) is more frequent than recombination between clades (Didelot et al., 2011). In *N. meningitidis*, the restriction modification systems appear to promote genetic exchange among closely related genotypes and to lower genetic exchange among strains that belong to different CCs or related species (Caugant and Maiden, 2009), leading to 'unobservable recombination'. The phylogenetic clades (near-clades) revealed by WGS in this species are associated with specific restriction modification systems that modulate homologous recombination (Budroni et al., 2011). In *S. pneumoniae*, some worldwide CCs are not transformable, and therefore do not have access to the DNA from other genotypes (Henriques-Normark et al., 2008).

In viruses, Holmes (2009, 2013) and Simon-Loriere and Holmes (2011) consider that recombination is linked to genomic architecture, and that obstacles to recombination are mechanistic and not only due to purging selection.

The in-built mechanisms that restrain recombination hypothesized in the PCE model therefore do exist in bacteria and probably in viruses. In fungi, it has been proposed that, although the sexual machinery was present in fungi, they 'retain sex' and their populations are often clonal (Heitman, 2006). Taylor (2015) defends the view that intrinsic obstacles to recombination exist in fungi.

The hypothesis of a 'clonality/sexuality machinery' in parasitic protozoa and in other pathogenic microorgansism (Tibayrenc and Ayala, 2012) still

has to be explored. However, we consider unlikely that natural selection be the only, or even, the main mechanism explaining the PCE features.

10. IDENTICAL MULTILOCUS GENOTYPES ARE A RELATIVE NOTION: IMPLICATIONS FOR THE SEMICLONAL/EPIDEMIC CLONALITY MODEL

We have long called attention to the fact that MLG monomorphism depends highly on the level of resolution and molecular clock of the marker concerned. Clonal genotypes should be better considered to be families of closely related clones. We have proposed the concept of 'clonet' (Tibayrenc et al., 1991b) to refer to those sets of stocks that appear to be identical with a given set of genetic markers in a basically clonal species. Using markers with a higher resolution is bound to reveal additional variability within each clonet. An illustrative example of clonet is the *L. infantum* MLEE MLG MON1, which proves to be genetically heterogeneous by the use of microsatellites (Ferreira et al., 2012). A more recent, striking case is the so-called *L. donovani* core group in India and Nepal, which is monomorphic with microsatellites, while it exhibits a strong genetic structuration into six congruent monophyletic groups with SNPs (Imamura et al., 2016). The clonet feature does not invalidate population genetics tests based on MLEE and microsatellite polymorphisms. As a matter of fact, considering as a clonal genotype what is actually a family of closely related clones leads only to a lack of resolution of the tests, but does not bias them. However, it specifically questions the approach proposed by Maynard Smith et al. (1993) for exploring clonality in micropathogens, namely, the so-called 'epidemic clonality' model, which is analogous to the 'semiclonal model' (Maiden, 2006). It proposes that LD in certain bacterial populations is explained by the occurrence of occasional bursts of 'epidemic', ephemeral clonal genotypes in an otherwise recombining species (Fig. 3). It is an exact mirror image of the 'fireworks' model proposed by Avise (2015) for clonal vertebrates (occasional 'fireworks' of ephemeral outcrossing events in a clonal species). In the approach proposed by Maynard Smith et al. (1993), repeated MLGs are considered to result from such recent clonal expansions. When counting each MLG only once, one should discard this bias and see whether the species is clonal (LD persists) or 'epidemic' (LD disappears). There is a bias in the approach, in that discarding many individuals many times reduces the sample size, which leads to an increased risk of statistical type II error. More concerning, the clonet concept shows that 'identical' MLGs can be genetically

very diverse; in other words, their common ancestor could be ancient. It can be therefore arbitrary to consider them as recent clones. We have seen earlier that the seimiclonal/epidemic clonality model in *N. meningitidis* is questioned by the PCE approach. The bias suggested by the clonet concept could be an explanation of it.

11. GENOMICS AND THE PREDOMINANT CLONAL EVOLUTION MODEL

Ramírez and Llewellyn (2015) urged us to wait for the forthcoming wealth of high-resolution data before considering whether it is appropriate to refine or reiterate our PCE hypothesis. We will certainly try and refine it, as we have done, especially in the recent years. However, in the light of the already available abundant genomic data, we do reiterate the PCE model. As a matter of fact, nothing in WGS and massive use of SNPs challenges the model. On the contrary, a large amount of data reinforce it. They have already been exposed, and only some of them will be briefly recalled in the following discussion.

WGS has made it possible to reveal hidden deep phylogenies and to confirm near-clade patterns in *N. meningitides* (Budroni et al., 2011), *S. pneumoniae* (Chewapreecha et al., 2014; Croucher et al., 2011; Muzzi and Donati, 2011) and *S. pyogenes* (Beres et al., 2010). It has shown a fine RD pattern in the ubiquitous near-clade H58 of *S. typhi* (Wong et al., 2011). In *E. coli*, WGS has confirmed the long-lasting near-clades evidenced years ago by MLEE (Chaudhuri and Henderson, 2012; Tenaillon et al., 2010) and has shown that the high rate of recombination in this species did not destroy its clonal population structure (Bobay et al., 2015). WGS in *M. tuberculosis* has evidenced 6 'phylogenetic groupings' (near-clades) that were not visible with classical markers (Achtman, 2008; Comas and Gagneux, 2009; Smith, 2012).

When parasites are concerned, genomic data have shown that *T. brucei gambiense* I was strictly clonal (Weir et al., 2016). WGS makes it possible to distinguish strict asexuality from PCE with occasional bouts of genetic exchange, which is difficult or impossible with classical markers and population genetic analysis (Weir et al., 2016). This makes it possible to refine the PCE model, not to refute it. Genomic data have evidenced a monophyletic near-clade in Turkish populations of *L. infantum* and have shown that the population structure within this near-clade was 'primarily clonal' (Rogers et al., 2014), which shows that 'multiple' later genetic exchange events (Ramírez and Llewellyn, 2015) do not prevent clonality and an RD pattern.

Genomics has revealed a fine RD pattern in the *L. donovani* 'core group', which is monomorphic with microsatellites (Imamura et al., 2016). In *P. falciparum*, WGS has shown that Papua New Guinea populations of the parasite were highly inbreeding in spite of abundant transmission (Manske et al., 2012) and that West Cambodian populations were 'essentially clonal' (Miotto et al., 2013). Lastly, WGS has made it possible to support the hypothesis of widespread aneuploidy in *Leishmania* (Downing et al., 2011) and *T. cruzi* (Reis-Cunha et al., 2015), which is highly relevant to the PCE model and supports it.

12. RELEVANCE OF THE PREDOMINANT CLONAL EVOLUTION MODEL FOR TAXONOMY AND APPLIED RESEARCH

The near-clade concept makes it possible to revisit the species concept in micropathogens. Either already described species and subspecies can be equated to near-clades from an evolutionary point of view or presently identified near-clades could constitute the starting point for describing new species or subspecies. The flexible phylogenetic approach based on the congruence criterion relaxes the demands of a strict cladistic analysis, which is indispensable, since some recombination occurs in almost all micropathogen populations. The near-clade model therefore gives a convenient and flexible framework for describing new species and subspecies, based on the phylogenetic species concept (Cracraft, 1983). The use of the mixiologic species concept (Mayr, 1940) has been tentatively proposed for some micropathogen species on the basis of occasional genetic exchange (Cacciò and Sprong, 2010; Dykhuizen and Green, 1991; Ngamskulrungroj et al., 2009; Voelz et al., 2013). However, genetic exchange in most micropathogens does not play the same evolutionary role as in metazoa, and cannot be retained as a valid criterion for species definition in their case.

In eukaryotic microbes, the sibling species *C. neoformans* and *C. gattii* can be equated to near-clades (Tibayrenc and Ayala, 2014b), since hybridization is possible between them (Bovers et al., 2006, 2008).

It is also most probably the case for several, if not most, *Leishmania* species, since hybridization is widespread in this group of parasites (Odiwuor et al., 2011; Ravel et al., 2006). From an evolutionary point of view, many presently described *Leishmania* species are equivalent to the *T. cruzi* near-clades.

The case of *L. killicki* and *Leishmania tropica* gives the opportunity to discuss the use of the near-clade concept to describe new subspecies. *L. killicki* appears

to be a genetic subdivision (near-clade) of *L. tropica* (Chaara et al., 2015a,b), which has led the authors to consider that this species was not valid. They have proposed to rename it '*L. killicki* syn. *Tropica*'. As a matter of fact, if one taxon is included in another one, one cannot give to both an equal taxonomic level. However, the fact that *L. killicki* corresponds to a clearly identified near-clade could give a ground to describe it as a subspecies, *L. tropica killicki*, since its epidemiological specificities could justify it.

We have proposed that new entities described in the genus *Plasmodium* may correspond to the evolutionary definition of near-clades and have warned against the tendency to equate them to new species without further evidence. Even their status as near-clades has to be ascertained, since their description most times is based on limited population and genetic samples (Tibayrenc and Ayala, 2014a).

In the genus *Pneumocystis*, several species have replaced the former species *Pneumocystis carinii*. Description of these species is mainly based on the criteria of host specificity and phylogenetic divergence. However, this specificity is not strict. The *Pneumocystis* 'species' might well be equated to near-clades, although data are far less abundant than for *C. albicans* and *C. gattii/C. neoformans* (Tibayrenc and Ayala, 2014b).

In *G. intestinalis*, the 'assemblages' that subdivide the species, which are perfectly equivalent to near-clades (Tibayrenc and Ayala, 2014b), have not been given a species status until now, although this has been proposed (Xu et al., 2012). Once never-clades have been identified and delimited (which is the case for the 'assemblages' in *Giardia*), their description as new species is a matter of convenience, if the concerned specialists (such as the *Giardia* scientific community) find it desirable and informative (Tibayrenc and Ayala, 2014b).

The *B. cereus* group is traditionally divided into six species, namely, *B. anthracis*, *B. cereus*, *Bacillus mycoides*, *Bacillus thuringiensis* and *Bacillus weihenstephanensis*. MLST analysis of 667 strains shows that the *B. cereus* group is actually composed of three major clades, which show no strict concordance with the six species, although all *B. anthracis* strains are in clade 1, most of the *B. thuringiensis* are in clade 2, and clade 3 is composed of all the *B. mycoides* and *B. weihenstephanensis* strains (Didelot et al., 2009a). Since there is recombination among the three clades (Didelot and Falush, 2007), they perfectly fit the definition of near-clade. These three near-clades therefore do not show a strict concordance with the six species described in the *B. cereus* group.

The three species *N. meningitidis*, *N. gonorrhoeae* and *N. lactamica*, which are discriminated by MLST, but among which some recombination goes on

(Achtman and Wagner, 2008; Bennett et al., 2007; Hanage et al., 2005), can be equated to near-clades.

The case of the mitis group of *Streptococcus* (the species *S. pneumoniae*, *pseudopneumoniae*, *Streptococcus mitis* and *Streptococcus oralis*) is quite comparable. The four species exhibit phenotypic specificities, and correspond to clear MLST clusters that can be equated to near-clades, since recombination is not totally absent among them (Fraser et al., 2007).

In addition to revisiting the problem of species and subspecies in micropathogens, the PCE model makes it possible to identify relevant units of analysis: clonets and near-clades, for molecular epidemiology (strain typing, epidemiological follow-up, tracing of genes of interest), clinical studies as well as vaccine and drug design. As an example, it has been recommended to take the *T. cruzi* near-clades ('DTUs') as a basis for all studies dealing with drug research concerning this parasite (Zingales et al., 2014).

These units of analysis, as ascertained by the PCE approach, display the characteristics that make them relevant for applied studies (discreteness, stability in space and time). The clonets (clonal MLGs) and near-clades can be characterized by one or a few genes, because of LD (indirect typing).

Lastly, clonets and near-clades can be conveniently used for experimental evolution studies. We have used *T. cruzi* near-clades to explore their specificity for gene expression (Machin et al., 2014; Telleria et al., 2010).

13. CONCLUSION

To our knowledge, this is the first time that informative comparisons have been made concerning evolutionary traits of all kinds of micropathogens (parasitic protozoa, fungi, bacteria and viruses). Until now, with few exceptions (Xu, 2004, 2006b), this field of research has remained strongly compartmentalized. Our broad survey has made it possible to show that micropathogens' population structures display striking similarities that appear to be major evolutionary strategies of these organisms, probably towards adapting to the parasitic way of life. As noted by Shapiro (2016), pathogens are more likely than free-living bacteria to undergo clonal expansions. Moreover, clonality appears to be a stable trait in given populations. 'Clonal populations tend to stay clonal' (Shapiro, 2016). It remains to be determined whether these features are ancestral or rather, constitute a remarkable case of convergent evolution.

The evidence for PCE is not the same among all micropathogens we have surveyed. Several species show a very typical PCE pattern, based on

strong and diversified evidence. This is the case for *C. albicans*, the *C. neoformans* complex, *E. coli*, *G. intestinalis*, *L. infantum*, *M. tuberculosis*, *S. aureus*, *T. gondii*, *and T. cruzi*. This is to the point that, through blind lectures, population genetic data from, let us say, *G. intestinalis*, *T. cruzi*, *C. neoformans* and *E. coli* could be confounded.

The PCE approach has made it possible to confirm that *P. falciparum* and *P. vivax* are capable of clonal propagation. However, these parasites undoubtedly undergo frequent recombination, which implies that PCE features (LD and near-clades) are unstable in them. Yet, it is the case that clonality in them introduces a major populational stratification feature that should be taken into account in all studies dealing with these parasites' genetic variability. It remains to be determined whether clonality in *Plasmodium* is due to 'starving sex' (lack of opportunity for mating) or to 'in-built clonality', or a combination of both.

It is remarkable that species previously considered highly recombining (*N. meningitidis*, *S. pneumoniae*) display typical PCE features. The advent of powerful technologies (WGS) has made it possible to evidence in these species the existence of deep phylogenies, which confirms the stability of the near-clades at an evolutionary scale.

Viruses constitute a special case in our study. Although several species show typical PCE features (near-clading and RD patterns), the evolution rate of these pathogens is considerably faster than that of cellular organisms, which implies that their genome undergoes a very rapid turnover. Still the fact remains that viral near-clades can persist for many years, as evidenced by retrospective studies or analyses of ancient collections of strains.

For many other species surveyed here (Table 1), evidence for PCE remains partial and has to be confirmed by more detailed studies.

Lastly, PCE criteria make it possible to settle the PCE 'boundary' at the clonality/recombination threshold, beyond which clonality definitely counters the effects of recombination, and the near-clades are bound to diverge more and more. It is probable that beyond this threshold, micropathogens do not exhibit a homogeneous strength of PCE. For example, *N. meningitidis* and *S. pneumoniae* probably undergo more recombination than *E. coli* and *S. aureus*. Our point is that they seem to have crossed the clonality threshold beyond which the near-clades will never get erased by recombination.

Apart from allowing the spread of successful MLGs and avoiding the recombinational load, a major feature of the PCE model is the generation of near-clades. Through this mechanism, which can be seen as an incipient speciation, or incomplete speciation, micropathogens could explore new

ecological niches through phenotypic specialization of the near-clades, including within a given host. It is also possible that mixed infections with two or more near-clades play a role in the pathogenicity of some microbial species. All these hypotheses are highly falsifiable.

PCE provides highly falsifiable assumptions that can be easily tested since our approach relies on a close analysis of a large amount of rough data with as few working hypotheses and models as possible. The PCE model establishes clearly defined predictive properties (relevant units of analysis that are stable in space and time) of high interest for evolutionary and applied studies dealing with pathogens.

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