Reproductive clonality of pathogens: A perspective on pathogenic viruses, bacteria, fungi, and parasitic protozoa

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We propose that clonal evolution in micropathogens be defined as restrained recombination on an evolutionary scale, with genetic exchange scarce enough to not break the prevalent pattern of clonal population structure, a definition already widely used for all kinds of pathogens, although not clearly formulated by many scientists and rejected by others. The two main manifestations of clonal evolution are strong linkage disequilibrium (LD) and widespread genetic clustering ("near-clading"). We hypothesize that this pattern is not mainly due to natural selection, but originates chiefly from in-built genetic properties of pathogens, which could be ancestral and could function as alternative allelic systems to recombination genes ("clonality/sexuality machinery") to escape recombinational load. The clonal framework of species of pathogens should be ascertained before any analysis of biomedical phenotypes (phylogenetic character mapping). In our opinion, this model provides a conceptual framework for the population genetics of any micropathogen.

molecular epidemiology | infectious disease | selfing

In the last two decades, the population genetics and evolution of pathogens have received much deserved attention. Impressive progress has been achieved through the development of whole-genome sequencing (WGS), bioinformatics, and other powerful molecular technologies. This progress has made it possible to explore, in depth, the central question of genetic exchange in pathogens, the issue of clonality vs. sexuality, which emerged in the 1980s, both in parasitic protozoa (the "clonal theory of parasitic protozoa") (1–3) and in bacteria (4–6). We seek to update the terms and interpretations of the controversy. Compartmentalization among researchers working on different pathogens has resulted in misinterpretations, semantic confusion, and different methods of analysis that often reflect idiosyncratic practices among different scientific communities, rather than distinctive evolutionary features.

We analyze population genetic data for bacteria (48 species) (4–82), fungi and yeasts (9 species) (83–93), parasitic protozoa (21 species) (1–3, 94–162), and viruses (11 species or categories) (163–188) (Table S1). There are striking evolutionary similarities among different kinds of pathogens, which are obscured by compartmentalization. We propose ways of consolidating the different approaches and of exploring whether similar evolutionary strategies represent ancestral characters or convergent evolution. We summarize the implications for applied research (including taxonomy, molecular epidemiology, medical characters, and experimental evolution).

Definition of Clonal Evolution: Restricted Genetic Recombination

In our early papers dealing with the clonality/sexuality issue in parasitic protozoa and fungi (1–3), we advanced an unambiguous definition of clonality/clonal evolution. It did not refer to the cytological mechanism of reproduction, but rather to the population structure that results from an absence or restriction of genetic recombination. Clonal population structure was defined in terms of genetic clonality, not cytological or physiological clonality. Moreover, we insisted that clonality does not mean total absence of recombination, but that it is too rare to break the prevalent pattern of clonal population structure (123, 189–194). A similar view was advanced for bacteria (4–6).

This definition is widely accepted in papers dealing with the population structure of pathogens: to wit, papers dealing with (*i*) general population genetics (195–197); (*ii*) all kinds of pathogens (89, 198); (*iii*) bacterial species (7, 12, 13, 30, 35, 72, 199–204), including *Borrelia* (18), *Burckolderia pseudomallei* (19, 20), *Campylobacter jejuni* (21), *Escherichia coli* (26, 27), *Legionella pneumophila* (36, 38), *Mycobacterium tuberculosis* (45, 48), *Neisseria meningitidis* (51, 54, 62), *Pseudomonas aeruginosa* (63, 65), *Pseudomonas syringae* (66), *Salmonella typhi* (68), *Staphylococcus aureus* (68, 70), *Streptococcus epidermitis* (70), *Streptococcus pneumoniae* (77), *Xanthomonas campestris* (81), and *Yersinia pseudotuberculosis* (82); (*iv*) fungi (83, 88, 205); (*v*) parasitic protozoa, including various apicomplexa species (95), *Cryptosporidium parvum* (97), *Giardia* species (98, 99, 101), *Leishmania chagasi* (113), *Perkinsus marinus* (119), *Plasmodium falciparum* (121, 124, 130), *Toxoplasma gondii* (83, 136, 139–142), *Trypanosoma brucei gambiense* (146), and *Trypanosoma cruzi* (149, 154, 155); and (*vi*) viruses (172, 178, 206).

The cited papers consider restrained recombination as the main criterion for clonality, without necessarily implying that the species under study are clonal. Often, clonality, lack or scarcity of recombination, and asexuality are appraised to be interchangeable notions (12, 30, 60, 63, 65, 71, 83, 87, 89, 91, 96, 98, 99, 130, 195–198, 207, 208). We have argued a related notion (i.e., a consequence of restrained recombination) (193, 194), namely that clonality obtains wherever the offspring cells show multilocus genotypes (MLGs) that are identical or nearly so (as ascertained using genetic tools of adequate resolution) to those of the parental cells (112, 195, 197, 208–210).

Clonality vs. Selfing/Homogamy

We have repeatedly considered selfing/strong homogamy as particular cases of clonality (2, 3, 123, 161, 190, 193, 194) and not as distinct evolutionary processes, a consideration shared by authors dealing with fungi (88, 205, 211) and parasitic protozoa (95, 96, 99, 109, 119, 121, 124, 130, 133, 135, 137, 139, 142, 148). Other authors, mainly working with *Leishmania* species (107, 111, 115, 116, 212) but also with *T. cruzi* (153) and fungi (87, 197), assert,

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however, that clonality should be distinguished from selfing/inbreeding and that clonality should be limited to "strict" clonality, that is, mitotic propagation. This restricted meaning is a matter of definition. Our opinion is that the broader definition of clonality, proposed by us and shared by most authors, which includes selfing/homogamy, should be privileged for several reasons: (*i*) The evolutionary consequences of strict clonality and strong selfing/homogamy are similar, in that they lead to linkage disequilibrium (LD) and the propagation of unchanged MLGs; (*ii*) restrained recombination is the important consideration in applied research, such as molecular epidemiology (strain tracking and typing) and tracing of genes of interest; (*iii*) although homogamy/selfing occurs in bacteria (51, 58, 199, 200, 213) and probably in viruses, most authors do not distinguish it from clonality; indeed, in the case of haploid organisms, the distinction is not relevant; (*iv*) most importantly, the population genetic means for distinguishing strict clonality from selfing/homogamy are questionable, because they rely on segregation tests designed mainly for diploid metazoa (107, 111, 115, 116, 148, 195, 197, 208, 209, 212, 214, 215). The common observation is a deficit of heterozygotes, although it is argued that strict clonality (mitotic propagation) should lead to an excess, not a deficit of heterozygosity.

The following considerations are relevant to point *iv*: (*a*) Several causes, in addition to selfing/homogamy, can lead to heterozygote deficit, including genome-wide mitotic gene conversion (153, 211), null alleles, allelic dropout, homoplasy (of major consequence when microsatellites are concerned) (108, 137, 139, 216, 217), natural selection, and the Wahlund effect. The probability of these factors should not be evaluated separately (107, 111, 115, 116, 148, 212), because they are not mutually exclusive. (*b*) Deficit of heterozygotes, inferred from microsatellite analysis, clashes with single-nucleotide polymorphism (SNP) data for *Leishmania mexicana* and *Leishmania braziliensis* (106) and *T. cruzi* (160), where an excess of heterozygotes is recorded. However, selfing/inbreeding should also lead to a heterozygote deficit for loci showing SNPs. (*c*) In *Giardia intestinalis/duodenalis*, heterozygote deficit has been considered an indication of ancient clonal evolution by purifying selection/gene conversion, whereas heterozygote excess is evidence for a recent sexual event/hybridization (98). (*d*) Experiments in *Daphnia pulex*, which is strictly asexual, have shown that a loss of heterozygosity by mitotic recombination is 1,000 times more frequent than an accumulation of divergent mutations (218). (*e*) Most important is the assumption of diploidy. In *Leishmania*, genomic studies strongly suggest that these organisms are highly aneuploid (106, 109, 117), which is also suggested by recombination experiments (219). Aneuploidy is inferred or strongly suspected in *Candida albicans* (211), *Phytophtora andina* (91), *G. intestinalis* (100, 103), *Trypanosoma congolense* (148), and *T. cruzi* (150, 151, 155). For the reasons given (*a*–*e*), conclusions about selfing/homogamy in parasites are questionable and should thus simply be considered as particular cases of clonality.

Clonality vs. Genetic Monomorphism

Sometimes "clonal" is understood, at least implicitly, as "genetically monomorphic" (10, 17, 45, 89, 119, 177, 196, 198, 220). This inference should be avoided because it is confusing when the issue at hand relates to the extent of recombination. Sexual species can be extremely monomorphic, due to recent origin and/or founder effects. Moreover, highly clonal pathogens, such as *T. cruzi*, exhibit considerable genetic polymorphism (1). In the absence of genetic polymorphism, no population genetic test or phylogenetic analysis is feasible, and it is unknown whether the species undergoes genetic recombination or not.

Main Features of Clonal Evolution

The main features of clonal evolution follow from its definition as "strongly restrained recombination." Most obvious is the propagation of persistent multilocus associations, which are stable in space and time and most significant in cases of widespread clones over several continents and many years. This is observed (*i*) in bacteria, *Bartonella bacilliformis* (15), *Bartonella quintana* (17), *L. pneumophila* (38), *M. tuberculosis* (44), *N. meningitidis* (49, 51, 58, 62), *P. aeruginosa* (63–65), *P. syringae* (66), *S. pneumoniae* (77), *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus* (80), and *X. campestris* (81); (*ii*) in fungi, *Candida dubliniensis* (87); and (*iii*) in parasitic protozoa, *T. cruzi* (1), *Leishmania donovani* complex (2), and *T. gondii* (139, 141, 142). These widespread clones are "superspreaders," or "successful clonal genotypes," highly relevant for epidemiological surveillance and molecular epidemiology.

Epidemiologically and evolutionarily highly relevant, but less intuitive, is the widespread occurrence of distinct clusters of genotypes (202), with an overdispersion of genetic distances between isolates (72), which are stable in space and time (sometimes, over several years and continents) and not imputable (or not only) to isolation by distance/time (Wahlund effect). Many bacteria show many examples of such population structure: *Bacillus anthracis* (10), *Bacillus cereus* (11), *Bartonella henselae* (16), *Borrelia burgdorferi* (18), *Campylobacter coli* (21), *Enterococcus faecium* (22, 23), *E. coli* (6, 25–27, 29, 31–33), *L. pneumophila* (37), *Listeria monocytogenes* (40, 41), *Listeria ivanoii* (40), *Mycobacterium bovis* (42), *M. tuberculosis* (42–45), *N. meningitidis* (46, 51–56, 58–62), *P. aeruginosa* (63–65), *P. syringae* (66); *Salmonella enterica* (67), *S. aureus* (23, 69, 70), *S. pneumoniae* (73, 74, 76, 78), *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus pseudopneumoniae* (73), *Streptococcus pyogenes* (79), *V. vulnificus* (80), *X. campestris* (81), *Yersinia pestis* (43), and *Y. pseudotuberculosis* (82). In fungi, such population structure is found in *C. albicans* (84, 86), *Cryptococcus gattii* (88), *Cryptococcus neoformans* (89), and *Fusarium oxysporum* (90). In parasitic protozoa, similar widespread population structure has been reported for *G. intestinalis* (99, 100, 104), *L. donovani* (108, 110, 111, 113, 216), *Leishmania tropica* (118), *T. gondii* (95, 134, 136, 139–141), *Trypanosoma brucei* (143–145), and *T. cruzi* (149, 151–154, 157, 160, 161, 194). In *P. falciparum*, clustering is apparent, although unstable in time (124). Widespread and stable clustering is found in viruses, particularly in RNA viruses, when taking into account the extremely rapid turnover of their genotypes. Structuring is clear in (*i*) RNA viruses: Chikungunya virus (169), coronaviruses (171), dengue virus (172–174), Ebola virus (175), Enterovirus echovirus (176), hepatitis C virus (177, 179), hepatitis E virus (180), HIV (170, 181), rabies virus (185, 186), and West Nile virus (187, 188); and (*ii*) DNA viruses: hepatitis B virus (164), Varicella zoster virus (168), and variola virus (167).

Three pertinent remarks about clustering in pathogens are that (*i*) in some cases, it concerns "microclustering," revealed by highly resolutive markers within species that appear poorly polymorphic with less resolutive markers (10, 42, 43) or within larger clusters (154, 157); (*ii*) it is observed in species, such as *N. meningitidis* (52, 58) or *S. pneumoniae* (13), in which genetic recombination is considered frequent; and (*iii*) because clustering is omnipresent in all kinds of pathogens, including viruses, bacteria, fungi, and parasitic protozoa, it is misleading to describe new species only on the basis of the clustering/phylogenetic background, as this would lead to a cumbersome inflation of the number of named "species."

Criteria to Identify Clonality/Restrained Recombination

Clear criteria for defining clonal population structure are sorely needed. Examples negating clonality use subjective expressions such as "strong influence of recombination" (15), "highly recombining" (20), "far from being a clonal species" (36), "extensive genetic exchange" (58), and others. We propose to put the cursor of clonality where two main, complementary, linked, criteria are the case: (*i*) strong LD and (*ii*) clear phylogenetic signal.

i) LD, or nonrandom association of genotypes at different loci, was circumstantial evidence for clonality in early papers (1–6). It is considered unreliable to explore the mating system of

pathogens in several papers (12, 195, 208, 209, 212, 214, 215). Apart from the classical biases that could lead to LD (Wahlund effect) (3), the reason invoked is a lack of resolution (147). Sometimes, the clonality hypothesis, based on LD analysis, is considered spurious due to a lack of resolution of the genetic markers used (111, 212). However, a lack of resolution favors the null hypothesis of panmixia, by increasing the risk of type II error. Segregation tests that explore allele distribution at given loci are often preferred to LD analysis, although, with eukaryotic pathogens, the results of such tests are questionable (section on *Clonality vs. Selfing/Homogamy*).

Criticisms against LD statistics are exaggerated. First, when the consensus definition of clonality is accepted (strongly restricted genetic recombination), LD is the adequate statistic to explore it, because it has been precisely designed to ascertain recombination or a lack thereof; segregation tests are useless for this purpose. Second, evidencing the presence and stability in space and time of multilocus associations is the very goal of molecular epidemiology (strain tracking) (193). LD tests are adequate tools; segregation tests are not. Third, if a suitable range of markers is used, LD becomes extremely powerful to provide evidence of departures from panmictic expectations and restrained recombination (2, 3). Fourth, LD analysis can be used whatever the ploidy is of the organism, even if it is unknown, and even without identifying individual alleles (190). Therefore, LD is more robust than segregation analysis, because it requires fewer working hypotheses.

Several authors consider LD reliable for exploring the population structure of pathogens and other organisms (37, 48, 53, 59, 62, 66, 70, 81, 136, 201, 205, 207, 213, 221). A widely used measure of LD is the "index of association" (I_A) test (203). In previous investigations, we proposed the use of tests (2, 3) that yield equivalent results as I_A statistics (123). Other LD tests are the *g* test (2, 3) or correlation between independent sets of genetic markers and the composite genetic equilibrium test (205), an adaptation of the I_A test for diploid organisms.

ii) Strong phylogenetic signal. To evaluate the strength and persistence, in space and time, of clonal evolution, LD analysis should be complemented by a strong phylogenetic signal, which provides evidence of clonal population structure. Tests, such as BAPS (73), CLONALFRAME (11), and STRUCTURE (67), are able to detect clear structuring and provide phylogenetic signal. Standard phylogenetic tests can also be used.

Whatever the tests used, evidence for strong phylogenetic signal should not rely on strict cladistic expectations, because some recombination is likely present in most species of pathogens. Rather, the evidence should be based on a flexible application of the congruence principle (222), which asserts that additional evidence will lead to convergent conclusions, whenever the working hypothesis is valid. Approaches based on the congruence principle can be, for example, based on our *g* test (2, 3). If two or more different sets of genetic markers yield converging phylogenies, they reveal a clear phylogenetic signal. This has been observed in (*i*) bacteria: *B. bacilliformis* (15), *B. henselae* (16), *E. coli* (25, 31), *L. monocytogenes* (41), *M. bovis* (42), *M. tuberculosis* (45, 48), *N. meningitidis* (52, 55), *P. aeruginosa* (63, 64), *S. aureus* (70), and *S. pneumoniae* (75); (*ii*) fungi: *C. albicans* (84, 86) and *F. oxysporum* (90); (*iii*) parasitic protozoa: *L. donovani* (109, 110, 114), *T. gondii* (136), and *T. cruzi* (3, 151, 152, 158, 159); and (*iv*) viruses: Echovirus (176).

Sometimes, discriminating markers reinforce the phylogenies. For *N. meningitidis* (54), *S. pneumoniae* (78), and *B. pseudomallei* (20), bacteria assumed to frequently recombine, WGS established clear deep phylogenies, which was not possible with multilocus sequence typing (MLST).

The "extended *g* test" we proposed (190) looks for congruence between different loci for the same marker. Looking for congruence between different loci by MLST is analogous to the extended *g* test. However, expecting exactly the same evolutionary history at all loci (60) is an excessive demand for pathogens, because some recombination occurs in most, if not all, species. If the phylogeny gets reinforced as additional loci are considered, it is a manifestation of a strong phylogenetic signal and, hence, of clonal evolution. According to the "semiclonal model" (60), recombination should not erase the impact of clonality in the short term (years or decades), but it would in the long term. It is analogous to the "epidemic clonality model" (203). A clear phylogenetic signal, revealed by plotting together the data from different MLST loci, is, therefore, not compatible with this model. For example, by concatenating the sequences from different MLST loci, one "buffers" the effects of limited recombination (81, 223). More than "masking" recombination (142), this approach reveals a hidden phylogenetic signal. Another approach, based on the congruence principle, is the use of various tests and software on the same dataset (20, 26, 81, 82). If there is a clear phylogenetic signal, the results should converge.

When bacteria are concerned, a phylogenetic signal should first be sought in the core genome, rather than in the dispensable genome (28). This process evidences a "clonal backbone" (66), "vertically inherited DNA" (31), or "genomic signature" (213). Genes under selective pressure may distort the phylogeny of clonal descent (201). Whatever the organism, phylogenies should be explored using genes that are not subject to strong selection.

Sampling Strategies

Appropriate sampling is crucial for exploring population structure. Some authors recommend starting with the smallest possible scale of time and space, such as, for example, individual organs (196, 199, 214), to avoid, as much as possible, the Wahlund bias. Sampling at the smallest scale possible is a wise strategy when focusing on the precise mating system of a species. However, when exploring population structures in the long term, we recommend also using the opposite strategy, i.e., taking a birds-eye view of genetic variability over years and continents, from different hosts and ecosystems (190). This complementary strategy yields a robust picture of a species population structure in the long run. Consistent patterns observed in different places and years apart are a strong indication of stable intraspecific genetic variability. Retrospective studies of old publications and/or ancient collections of strains are useful. For example, recent studies (25, 27, 32) have corroborated the evidence from ancient studies of *E. coli* (4, 6), indicating a strong stability of the genetic clustering for this species. The same comment applies to *N. meningitidis* (52, 55, 59, 62) and *T. cruzi* (1, 149, 159, 161, 191). Also, the population structure of *L. tropica* in Asia has been stable for at least 55 y (118). Studies of *P. aeruginosa* over 125 y have shown the persistence over many years of worldwide clones (63). Chikungunya virus phylogroups and subgroups show strains that have been sampled over many years (169). "Clade" distribution of dengue virus in Vietnam appeared to be stable from 2003 to 2008 (173).

Another point is the number of isolates that should be obtained from a given host at a given time. A simulation study (224) suggests that sampling only one isolate per host in a clonal species would mimic recombination, whereas the contrary would obtain in a recombinant species. This claim has not been confirmed by a study on *C. albicans* (85), which involved 5–10 isolates per patient. Plotting together the results from all isolates led to lowering the level of LD. A microsatellite study on *T. cruzi* (155) has shown that original, noncloned isolates yielded the same results (heterozygosity, LD) as individually cloned stocks obtained from the same isolates.

A widely used sampling strategy is to apply population genetic tests, first on the whole sample and then within each of the genetic clusters that subdivide it (88, 115, 148, 157). The working hypothesis is that gene flow is inhibited between the clusters, but

not, or less, within each of them. This strategy is informative, but has a risk of type II error. Apparent lack of departure from panmixia could be due to lack of sufficient genetic information. With this strategy, evolutionary levels are shifted. Consequently, genetic markers with a "faster molecular clock" and higher evolutionary/mutation rate should be used. MLST of individual sequence types (ST) in apparently monomorphic bacterial species using increasingly finer genetic markers (WGS/SNP) reveals additional microclusters within them (10, 43).

Semantics

The literature of pathogen population genetics abounds with a heterogeneous (and confusing) nomenclature that does not evidence different evolutionary entities, but rather the idiosyncrasies of different communities of researchers working on different pathogens. Some standardization is desirable, because similar names should be given to similar evolutionary entities, whatever the organism. Stable genetic subdivisions that manifest clonal evolution most probably represent similar evolutionary entities, whatever the species of pathogen. However, such entities have received various names, including (*i*) bacteria: clades (11, 21, 25, 29, 32, 52, 66, 67, 74, 76), clonal complexes (21, 23, 35, 51, 55, 61, 63–65), clonal lineages (204), clusters (10, 14, 37, 73, 77, 80, 81), genetic groups (81), genoclouds (24), groups (10, 31, 33, 44, 67), and lineages (7, 41, 43, 53, 67, 78, 200); (*ii*) fungi: clades (86), clonal lineages (91), and clusters (90); (*iii*) parasitic protozoa: assemblages (99–101, 104), clades (113, 125, 126), clonal lineages (95, 136, 139, 140), clusters (111, 143), discrete typing units (DTUs) (149, 151, 152, 156, 160, 161, 191), groups (127), and types (141); and (*iv*) viruses: clades (173, 176, 179, 180, 185–188), clusters (176), genogroups (176), genotypes (164, 174, 177, 179, 180), major genotypes (178), major lineages (187), and phylogenetic groups (171). In addition to this semantic confusion, some species described in *Leishmania* (102, 216), *Plasmodium* (125, 126, 129), and *Pneumocystis* (92, 93) likely amount to only genetic subdivisions resulting from clonal evolution/restrained recombination. Caution is also warranted in relation to identifying new *Plasmodium* species exclusively on the basis of phylogenetic grounds (132).

It is a major challenge to unify a nomenclature reflecting different views, traditions, opinions, and schools of thought of various "groups" of researchers, who tackle similar problems. The term clade should be dismissed for referring to the genetic subdivisions discussed in this article. A clade has only one ancestor and evolves separately from other clades. However, clonal evolution coexists with some horizontal transfer/genetic exchange in all pathogens. Genetic subdivisions are the result of clonal evolution, "purifying" selection, and some genetic exchange, processes that have variable consequences in different species. "Near-clades" could be appropriate for species in which a strong phylogenetic signal has been shown, but in which genetic recombination most probably occurs.

Clonal genotypes characterized by given genetic markers, whether MLST, SNPs, or multilocus enzyme electrophoresis, are misleadingly called "clones" (55, 64, 65, 69, 108). Using markers with greater resolution might reveal additional variability within clonal genotypes (10, 80, 142). The term "clonet," coined by us (194), is appropriate to describe genotypes characterized by a given genetic marker in a predominantly clonal species. It is inappropriate to consider microsatellite genotypes as real clones (208). Using more microsatellite loci or even more discriminating markers (e.g., variable surface antigen genes) might uncover additional variability within them. Long ago, we asserted (158) that only WGS would provide definitive evidence of real clones. This desideratum has now acquired currency (23, 28, 74, 201, 225, 226).

Evolutionary Significance of Clonal Evolution

Near-clades plus restrained recombination: Are they natural selection or in-built genetic mechanisms?

The occurrence of near-clades is widespread in many pathogens, including some considered as frequently recombining (21–23, 40, 55, 63, 78). Stable near-clades are observed as well in viral species (164, 165, 167–177, 179–181, 185–188), when taking into account the rapid turnover of viral genomes. In many species, recombination is severely restricted, leading to the propagation of stable multilocus associations (clones), which could be (*i*) the result of drastic natural selection acting on an otherwise quasi-freely recombining species or (*ii*) specific genetic mechanisms that inhibit recombination.

A major role for natural selection has been claimed to explain the persistence of clonal complexes and hypervirulent lineages in *N. meningitidis* (9, 51, 53, 55, 58, 62). Allegedly, natural selection is the factor shaping these persistent complexes and lineages over long time spans, wide geographical ranges, and diversified ecosystems in an otherwise close-to-panmictic species. This account is unlikely. Indeed, other authors have considered that the evolutionary pattern of *N. meningitidis* is compatible with neutral evolution (47, 54). If selection were the main factor explaining predominant clonal evolution, this would require the elimination in each generation of most possible MLGs, which would become a considerable genetic load (190). The clonal model that we propose (2, 3, 194) infers upstream inhibition of recombination (by in-built genetic mechanisms), rather than downstream elimination by "purifying selection," even though selection might play a role. We propose that pathogens have evolved inhibited recombination as an evolutionary strategy, mainly to avoid the recombinational load (disrupting favorable multilocus associations) (227, 228). The genetic systems that restrain recombination could be alternate allelic variants to genetic mechanisms favoring recombination, like a "clonality/sexuality repertoire toolkit," by analogy with the meiosis toolkit observed in *Giardia* and *Trichomonas vaginalis* (207). Systems that restrain recombination amplify the effects of natural selection (coadapted multigenic complexes) (222). Retaining sex (83) might yield fitness benefits.

In-built mechanisms for restraining recombination do exist in bacteria, in which the frequency of recombination is often inversely proportional to the genetic distance between genotypes (11, 14, 61, 200). There may be a threshold of genetic divergence beyond which a species would tend to clonality (72). "Illegitimate" recombination between DNA sequences that are excessively different could be hampered by mechanical obstacles (21, 36, 229). In *N. meningitidis*, restriction systems would be more similar between similar genotypes than between distant ones, favoring homogamy (51, 54, 58, 62). In *S. enterica*, differences in mismatch repair systems could inhibit recombination between genotypes that are too different (67). In-built genetic mechanisms for restrained recombination have been inferred in viruses (172, 206). Recombination happens more readily between more similar genotypes in adenoviruses (163) and picornaviruses (183). The "complexity hypothesis" states that recombination is inhibited for genes involved in complex, conserved processes (213). Experiments provide support for this hypothesis in viruses (166). Genetic mechanisms for recombination inhibition may also exist in fungi and parasitic protozoa, in which clonal evolution is widespread. Recombination seems easier between closely related genotypes pertaining to the same near-clade in both fungi (88) and parasitic protozoa (102, 104, 150). As in bacteria (51, 54, 58, 62) and viruses (163, 183), there may not be a clear-cut border between sexuality and clonality in some cases, but rather a progressive development of restrained recombination ("progressive clonality") (190). Are such hypothetical "clonality/sexuality genetic mechanisms" ancestral in pathogens or did they evolve independently in different groups of pathogens?

Recombination: What Is It Good for?

An evolutionary explanation of recombination is that it generates new multilocus combinations, some of which increase adaptation.

This hypothesis is retained for some viruses (166, 184, 186) and bacteria (30, 66). Other authors propose that recombination is a by-product of other processes, both in viruses (172) and in bacteria (213). The main role of recombination would be DNA repair (58, 195, 228). It has been even proposed that DNA repair could be the ancestral mechanism of the general process of sexuality (228). In *Giardia*, the meiosis machinery would be homologous to DNA repair bacterial genes (228). Recombination in viruses and bacteria might be ancestral for sexuality in eukaryotes (198, 228). By analogy, we suggest that genetic mechanisms to restrain recombination or, more generally, to keep a balance between clonality and recombination, could be ancestral from viruses to bacteria and to eukaryotic pathogens. Rather than a sexuality machinery (207), pathogens would be equipped with a "clonality/sexuality machinery" that would allow them to play on a double keyboard to face evolutionary challenges.

Clonal evolution complemented with occasional recombination is advantageous to many pathogen cases by stabilizing favorable multilocus associations. In *T. cruzi*, it is remarkable that hybrid genotypes stabilized by clonal evolution have become superspreaders in humans in the southern range of Chagas disease (102, 152, 156, 161, 194). A similar state of affairs may be the case in *Giardia* (98), *Leishmania* (102, 111, 194, 216), *Perkinsus* (119), *Toxoplasma* (134, 136, 139, 207), *C. neoformans (*89), *P. andina* (91), and *S. aureus* (70). The limited impact of recombination in a clonal species attenuates the drawbacks of long-term clonality (Müller's ratchet) (207).

Implications for Applied Research

The model of clonal evolution provides a backbone for applied research dealing with pathogens (molecular epidemiology, clinical studies, vaccine and drug design, and experimental evolution). The search for the phylogenetic signal and the design of a clear phylogenetic framework should precede investigation of the epidemiological/biomedical properties of the species under study, in a phylogenetic character-mapping (PCM) process (222, 230). "Placing evolutionary changes in their clonal context provides the power to relate phenotype to genotype" (ref. 46, p. 1). "Phylogenies provide the ability to predict phenotypic and genotypic traits" (ref. 217, p. 1010).

Flexible phylogenetic analysis based on genes that do not undergo strong selection constitutes an objective and reliable criterion, highly standardizable and applicable to any organism. This is not the case for ecological parameters (14), which are subjective and could be innumerable. Integrated approaches based on many characters (transcriptome plus proteome plus metabolome plus functional studies) (23) are difficult to interpret in phylogenetic terms. We advocate a hierarchical approach, with population genetics/phylogenetic analysis coming first.

Some Illustrative Cases

***N. meningitidis*.** This bacterium has been considered the paradigm of "semiclonal evolution" (60) or "epidemic clonality" (203). Recombination would erase the impact of clonality in the long run. This claim is contradicted by evidence of a clear phylogenetic signal (52, 54). Moreover, the "hyperinvasive lineages," confirmed by several kinds of genetic markers (55, 62), show remarkable stability over years and continents (51, 53, 55, 58, 62, 201) and a strong association with virulence phenotypes (61) and antigen polymorphism (53, 58–60). According to the criteria we are advancing, *N. meningitidis* should be considered definitely clonal, despite indications of frequent recombination (13).

***E. coli*.** The same is even more the case for *E. coli*, which, because of the retrospective analysis of pioneering studies (4, 6), has been shown to exhibit a striking persistence of near-clading features, corroborated by several kinds of genetic markers (25). Near-clades have been identified in environmental strains, questioning the limits of the species (29, 32). It is remarkable that biological phenotypes show a strong correlation with the genetic subdivisions evidenced by genetic markers (231). *N. meningitidis* and *E. coli* are both clonal, but the impact of recombination is stronger in the former (13).

***T. cruzi*.** The agent of Chagas disease shares many evolutionary features with *E. coli*. Whatever the ecosystem considered, *T. cruzi* populations always display strong LD (1–3, 158, 194), involving different genetic markers (151, 158, 159). Its subdivision into six stable near-clades or DTUs (191) has been corroborated (149, 152, 160, 161). Additional subdivisions can be evidenced by high-resolution markers (154, 157). It is not the case that recombination is "of little consequence" (156) in this parasite. On the contrary, it is most probable that it plays an important role on an evolutionary scale (194). However, it is not frequent enough to break the prevalent pattern of clonal evolution (1–3, 158, 194). Experimental recombination has been obtained (150), and genetic exchange in nature has been inferred in localized cycles (157), which illustrates two important facets of the clonal model: (*i*) Successful recombination experiments say nothing about the frequency of these events in nature, but show that the potentiality for genetic exchange is present; and (*ii*) the clonal model is compatible with limited genetic exchanges.

The evidence gathered to infer selfing/homogamy in the case of *Leishmania*, mainly heterozygote deficit, is present in *T. cruzi* and consistent with widespread clonality (155). Heterozygote deficit has been attributed to genome-wide mitotic gene conversion (153). It can be recalled that SNP characterization (160), contrary to microsatellites (155), shows an excess of heterozygotes in *T. cruzi*. Finally, although less definitive evidence is available, like for *Leishmania*, aneuploidy can be suspected in *T. cruzi* (151, 155), which casts doubt on tests based on the hypothesis of diploidy.

***Leishmania*.** In *Leishmania*, selfing/homogamy and strict clonality have been considered antithetical (107, 111, 115, 116, 212). We have explained here and in former studies (123, 193, 194) that selfing/homogamy is a particular case of clonality, not contrary to it. Moreover, convergent evidence for widespread aneuploidy (106, 109, 117) challenges the tests seeking to distinguish between selfing/homogamy and strict clonality, all based on the hypothesis of diploidy. We rank *Leishmania* species as predominantly clonal organisms (2, 3, 190, 192–194), a view shared by others (106, 109, 112, 118).

The observation of both heterozygote deficit and lack of LD in *Leishmania guyanensis* (116) is puzzling, because the two features should be incompatible. If genetic exchange is frequent in the populations surveyed, as proposed (116), lack of LD suggests high rates of recombination, not selfing. However, high recombination should lead to Hardy–Weinberg equilibrium at individual loci, not heterozygote deficit, whereas strong selfing should generate LD. A possible explanation is that the apparent low LD is due to the saturation of the marker, with a fast molecular clock generating too much variability, a frequent situation with microsatellites ("loci mutationally saturated") (108, 216, 217). This saturation could mimic sexuality/recombination (137): Even if the species is clonal, all, or almost all, isolates might exhibit a distinct MLG (220). As noted, it can be suspected that some or most of the many species of *Leishmania* (102, 216) are the evolutionary equivalents of near-clades described in species such as *T. cruzi* (161) or *G. intestinalis* (101, 104).

***P. falciparum* and *T. gondii*.** These two apicomplexan parasites were traditionally considered as obligatorily sexual. We proposed that both *P. falciparum* (2, 3, 123, 232) and *T. gondii* (3) undergo clonality in some cycles. In *T. gondii*, this hypothesis has been confirmed by studies showing the existence of near-clades (133–

142, 233), which have a hybrid origin further stabilized by clonal propagation. The impact of clonality and recombination is different between cycles; recombination is more frequent in South America (135, 137). In the *Toxoplasma* literature, selfing/homogamy is not distinguished from clonality.

In *P. falciparum*, the "panmictic hypothesis" (234) is now considered as "oversimplified" (83) and has been challenged by studies showing clonality in particular populations (120, 121, 130). The hypothesis that apparent clonality in *P. falciparum* could be explained by little opportunity for mating in low transmission cycles (120) has not been confirmed. Clonality is also recorded in high transmission cycles (121, 124). In contrast to other pathogen species, *P. falciparum* does not show a clear structuration into near-clades. Some structuring has been shown in Peruvian populations (124), but these subdivisions seem to be unstable, although they introduce a notable stratification of the parasites. It remains to be explored whether the groups and new species described in *Plasmodium* parasites mainly on the basis of phylogenetic criteria (122, 125–128, 131, 132) could be better considered as evolutionary equivalents to the near-clades of other species of pathogens.

Conclusion and Perspectives

We have advanced a clonal evolution model, based on a simple definition (restrained recombination on an evolutionary scale) and relying on clear criteria, namely strong LD and a distinct phylogenetic signal. It remains to be ascertained, by comparative studies, whether the evolutionary strategies discussed are also found in free-living microbes.

Our knowledge of the population genetics and evolution of pathogens will further advance through the use of powerful new technologies, including WGS, and innovative and effective computer programs for the analysis of data produced using these technologies. MLST has made a considerable contribution to this field of research (60). WGS, with a much higher resolution than that of MLST (68), is becoming a commonly used tool; and the sequencing of hundreds of strains might be within reach in the near future (217). WGS permits the definition of SNP markers (217, 225), provided that the phylogenetic discovery bias (also called ascertainment bias) is avoided (43, 199, 204, 217). WGS will make it possible to dissect the recombination/hybridization phenomena, which seem to be a major evolutionary strategy of many species of pathogens (91, 98, 163). WGS may soon become a standard approach for studying the molecular epidemiology of viruses (170), which are easy and rapid to sequence because of their small genomes. Finally, WGS might make it possible to ascertain whether the many cases of near-clading that we have discussed here correspond to similar evolutionary entities and also to disentangle the confusing terminology that hampers the development of the population genetics of pathogens.

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