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- generated fragment of α 1,3GT intron 2 to exon 4 ended at the start codon ATG as the 5' arm; Neo/pA coding region fused into the ATG of the 5' arm in-frame; a 9.6-kb PCR-generated fragment of α 1,3GT exon 4 to exon 7 as the 3' arm.
- 657A-111 1-6 cells were electroporated with pPL680 and cultured for 5 days. Cells were exposed to *C. difficile* toxin A (2 μ g/ml) (Techlab, Blacksburg, VA) in growth medium for 2 hours. Toxin A medium was then replaced with fresh growth medium. Medium was changed 7 and 11 days posttransfection to remove the detached cells. One colony (680B1) was harvested 13 days posttransfection for expansion and cryopreservation.
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Recent Expansion of *Toxoplasma* Through Enhanced Oral Transmission

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The global predominance of three clonal *Toxoplasma gondii* lineages suggests that they are endowed with an exceptional trait responsible for their current parasitism of nearly all warm-blooded vertebrates. Genetic polymorphism analyses indicate that these clonal lineages emerged within the last 10,000 years after a single genetic cross. Comparison with ancient strains (~1 million years) suggests that the success of the clonal lineages resulted from the concurrent acquisition of direct oral infectivity. This key adaptation circumvented sexual recombination, simultaneously promoting transmission through successive hosts, hence leading to clonal expansion. Thus, changes in complex life cycles can occur rapidly and can profoundly influence pathogenicity.

Toxoplasma gondii is a member of the phylum Apicomplexa, an ancient group of ~5000 species of parasitic protozoa that infect a wide range of vertebrates (1–3). Most closely related members of this group have complex two-host life cycles that alternate between definitive (sexual propagation) and intermediate hosts (asexual replication) (4). *T. gondii* is remarkable among this group for the extremely wide range of birds and mammals that serve as intermediate hosts, although sexual propagation is limited to members of the cat family (Felidae) (4). Toxoplasmosis is a major cause of foodborne illness acquired through ingestion of contaminated water or infected meat (5). Human infections, although globally widespread, are primarily subclinical, except in immunocompromised individuals in whom they are often severe (6–8). *T. gondii* has a highly unusual, clonal population

structure comprised of three widespread genotypes referred to as type I, type II, and type III (9–11). The genomewide rarity of polymorphisms within these lineages is suggestive of a recent and massive genetic “selective sweep,” in contrast to the otherwise ubiquitous and ancient nature of this group of parasites.

To estimate the age of *T. gondii* relative to other apicomplexans that form tissue cysts, we analyzed the small subunit (SSU) and internal transcribed spacer 1 (ITS1) regions of the ribosomal DNA cluster (12). Analysis of the SSU regions revealed that the genera *Toxoplasma*, *Hammondia*, and *Neospora* form a closely related triad, whereas other branches defined by *Sarcocystis tenella*, and the out-group *Eimeria tenella*, are quite distant (Fig. 1A) (13). Because there is no fossil record for the apicomplexans, we used the average SSU substitution rate calculated from a variety of taxa (14) to calculate the ages for lineages shown in Fig. 1. Notably, the node defined by the most recent common ancestor of *T. gondii*, *N. caninum*, and *H. hammondi* was estimated to be about 12 million years ago (Fig. 1A, table S1).

To provide greater resolution between these closely related taxa, we analyzed the ITS1 region, which has fewer structural constraints and is therefore typically more variable than the

SSU (Fig. 1B). Phylogenetic analysis of the ITS1 region clearly separated *N. caninum* from *T. gondii* and supported a paraphyletic origin for *Hammondia*, as described previously (15) (Fig. 1B). We sequenced the ITS1 region from a representative member of each of the three clonal lineages of *T. gondii* and found it to be identical at all 393 base pairs (bp), in agreement with a previous report (16) (Fig. 1B). By comparison, the amount of intraspecies sequence divergence in the ITS1 regions for several related Apicomplexa ranges from 1 to 6% (17–19). The lack of sequence divergence in the ITS1 region suggests that the major *T. gondii* lineages share a recent common ancestry.

Studies have shown that the three clonal lineages of *T. gondii* are highly similar, as estimated by restriction fragment length polymorphisms (10) and multilocus isoenzyme analysis (9). Furthermore, sequencing of individual genes indicates only 1 to 2% divergence (20–22). Sequencing of antigen-encoding genes established that the three clonal types are comprised of combinations of just two alleles at each locus, which indicates that they are the result of a recent cross between closely related parental strains (23). A small number (fewer than 5% of isolates) of recombinant strains, which have mixtures of the two-allele patterns, are also observed (10), and a few of these strains (<1%) contain unique polymorphisms (23–26). The latter strains are referred to here as “exotic.” Experimental crosses between different genotypes of *T. gondii* have demonstrated that genes are inherited in a Mendelian fashion and that many independent recombinants arise from a single cross (27, 28). Although recombination is apparently rare in nature, it might also be expected to give rise to a large number of distinct lineages. The predominance of just three clonal types in nature indicates that they have a trait or traits that allowed them to expand rapidly and dramatically after a recent origin.

To determine the relative divergence between *T. gondii* strains, we analyzed the frequency of single-nucleotide polymorphisms (SNPs) in noncoding regions consisting of 11 introns plus the ITS1 region that collectively constituted 4067 bp per strain. We compared the SNP frequencies among four type I, three type

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II, and three type III strains, isolated from both human and animal origins from different geographic sources (table S2). When the common dimorphic polymorphisms that define these lineages (23) were filtered out, we observed only two nonancestral changes within the collective 40,670-bp intron sequences from these 10 strains (table S3). We also analyzed a subset of exotic strains including MAS, CAST, and COUG: The first two exotic strains are most closely related to the type I lineage, whereas COUG is most closely related to type II strains. In contrast to the highly conserved nature of the clonal strain types, 42 separate nonancestral mutations were observed within the collective 12,201-bp intron sequences from these three exotic strains.

To estimate the time to most recent common ancestry (TMRCA) for *T. gondii*, we combined our data (table S3) with previous reports of SNPs within introns and antigen-coding regions from both clonal lineages and exotic strains of *T. gondii* (23, 25, 26). Collectively, these loci span 7 of the 11 *T. gondii* chromosomes (28). We estimated and used two rates for neutral mutations in the closely related apicomplexan *Plasmodium* (29, 30) and also generated an estimate of the rate of mutational change in introns of *T. gondii* genes by comparison with *N. caninum* (13). We considered a starlike phylogeny (genetic bottleneck followed by radiation) to be the best description of the current population structure of *T. gondii*. When we estimated the TMRCA of the predominant lineages using a Poisson model (30), we obtained an age of $\sim 10^4$ years using the rates of mutation based on *Plasmodium* (Table 1 and table S4). The rate of mutation for *T. gondii* introns was somewhat higher, which resulted in an even lower age estimate of $\sim 10^3$ years (Table 1 and table S4). In contrast, when we considered the exotic lineages with the same model, we calculated $\sim 10^6$ years for their origin on the basis of the *Plasmodium* mutation rates (Table 1 and table S4). This difference suggests that the exotic strains were not derived from the same cross that gave rise to the clonal lineages. Moreover, because the mutations observed in the exotic strains do not affect sites involved in the bi-allelic system (table S3), it is likely that they predate the cross that led to the predominant clonal types. Collectively, these results indicate that the clonal lineages are extremely recently derived from a single genetic cross, whereas the exotic strains represent more ancestral lineages as shown in Fig. 2.

The observed complete identity of isolates within a clonal lineage and the lack of genetic variation in *T. gondii* provides insight into how natural selection may have led to the current population structure. This pattern is consistent with a recent genetic selective sweep that allowed many loci to become simultaneously fixed via the hitchhiking effect (31). Such a model requires positive selection to act in the absence of recombina-

tion and presumes that avoidance of interbreeding was part of the biological process underlying the selective sweep.

One biological trait that is displayed by *T. gondii* but not by closely related parasites such as *Neospora*, *Sarcocystis*, and *Hammondia* is the ability to circumvent sexual reproduction in the definitive host (members of the cat family in the case of *T. gondii*) and to infect directly successive intermediate hosts after oral ingestion of tissue cysts (Fig. 1C, fig. S1) (4). If modern-day relicts of this ancestral state remain, differences in oral infectivity may be expected between *T. gondii* strains. Therefore, we tested representative clonal lineages versus

exotic strains by administering tissue cysts to mice orally, which simulates natural transmission between successive intermediate hosts (13). Tissue cysts of all three clonal lineages were infectious to mice when given orally, resulting in infection of 50 to 100% of mice (Table 2). Notably, type II strains were more infectious by the oral route, and they are the most predominant in nature (10). Although the exotic strain COUG was also infectious by the oral route, CAST was significantly less infectious, and MAS was not infectious at all by the oral route (Table 2). We also tested infection by intraperitoneal inoculation, which is not a natural route of infection, but provides a more

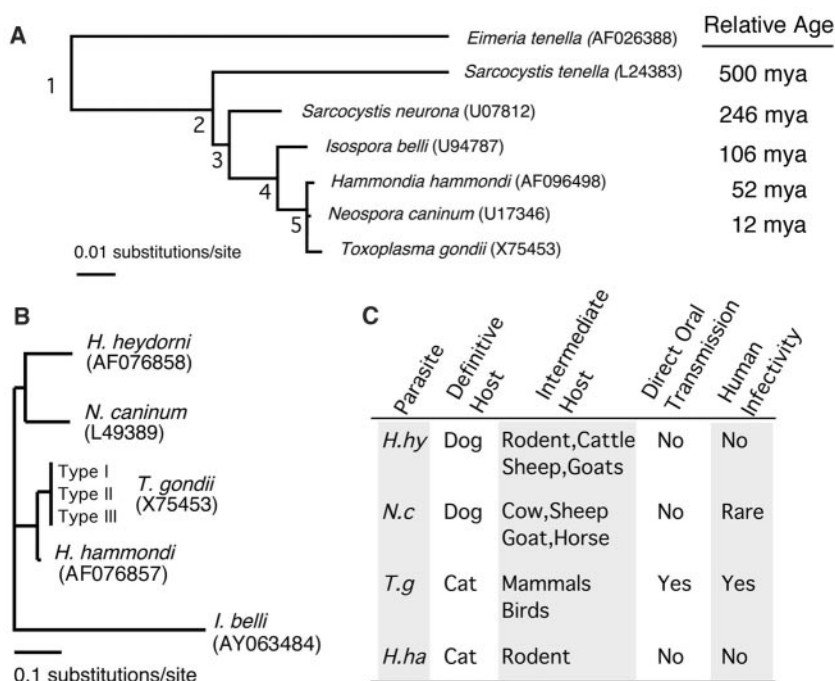


Fig. 1. Phylogenetic comparison of apicomplexans based on selected regions of the ribosomal DNA. (A) Neighbor-joining tree of apicomplexan SSU sequences. *E. tenella* was designated as the out-group and the remaining in-group taxa were considered monophyletic. Nodes numbered 1 to 5 were supported by bootstrap values of ≥ 85 . Relative age estimates from table S1 (13). (B) Neighbor-joining tree of the ITS1 sequences. (C) Life cycles and transmission characteristics for *T. gondii* and closely related taxa (4).

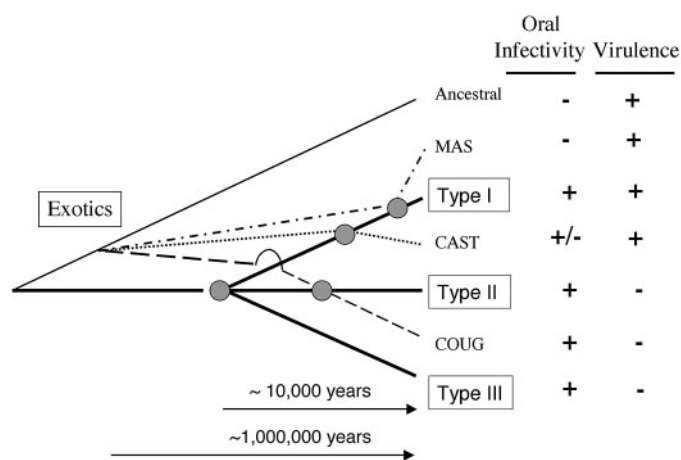


Fig. 2. Model for the origin of *T. gondii* lineages and direct oral transmission. Clonal lineages arose from a recent genetic cross, whereas exotic strains predate this origin but have subsequently introgressed. Age estimates from table S1. Oral infectivity is expressed by all the clonal lineages but only to a variable extent in exotics as defined in Table 1. Virulence as defined in (28).

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Table 1. Estimates of age in years since TMRCA for *T. gondii*.

Lineage	Sites (bp)	Mutations	Age in years since TMRCA for mutation rate		
			$1.7 \times 10^{-9*}$	$3.8 \times 10^{-9†}$	$1.9 \times 10^{-8‡}$
Clonal	90,764	2	1.3×10^4	6.1×10^3	1.1×10^3
Exotic	20,771	71	2.1×10^6	9.3×10^5	1.8×10^5

* (29). † (30). ‡ *T. gondii* intron rate (13).

Table 2. Oral versus intraperitoneal infectivity of *T. gondii* strains. Infectivity is defined as the combination of lethal and chronic infections as described (13, 28). The difference in combined oral infectivity of MAS and CAST versus that of the predominant clonal lineages (GT1, PTG, CTG) was significant (* $P < 0.001$) when analyzed using a chi-square test for independence.

Route of inoculum	Clonal lineages (%)			Exotic strains (%)		
	Type I (GT1)	Type II (PTG)	Type III (CTG)	MAS	CAST	COUG
Oral	47	100	47	0*	10*	100
Intraperitoneal	100	100	100	100	100	100

sensitive bioassay than oral challenge (32). All mice became infected when parasites were administered by intraperitoneal injection, which indicated that both clonal and exotic strains are infectious to mice when natural barriers are bypassed (Table 2). These results indicate that the predominant clonal lineages share the trait of direct oral infectivity via ingestion of tissue cysts, although this property is absent or only partially expressed in some exotic strains.

Even though absence of direct oral infectivity is an ancestral trait, not all the exotic strains examined here exhibit this tight restriction. This apparent discrepancy can be explained by closer examination of their genotypes (table S3), which reveals that they are mixtures of the two-allele system that defines the clonal lineages. This pattern strongly indicates that they are the result of introgression (i.e., recombinants formed by subsequent crossing with members of the clonal lineages) and hence they would be expected to exhibit variable expression of the recently derived trait of oral infectivity (Fig. 2). True ancestral strains are likely to be extremely rare in the environment because they are restricted in their transmission to the archetypal two-host life cycle. Nonetheless, the presence of this ancestral trait in some strains (e.g., MAS) may allow experimental analysis of the mechanism that underlies oral infectivity; components important for this trait would be excellent candidates for strategies to disrupt transmission.

Although the vast majority of *T. gondii* strains share a very recent common ancestry, they infest virtually all warm-blooded vertebrates (4), which have themselves diverged over the past tens to hundreds of millions of years (33). This dichotomy can be explained by a model where the current population pattern of *T. gondii* is the result of a single recent meiotic event that gave rise to a limited number of progeny that were newly endowed with the key

adaptation of direct oral infectivity (Fig. 2). Acquisition of direct oral infectivity would effectively by-pass sexual reproduction, thus supplying, in a single event, both a selective advantage and a means of fixing the entire genotype via the hitchhiking effect (31). Although acquisition of enhanced oral infectivity offers a plausible explanation for the unusual population structure of *T. gondii*, it is also possible that other key adaptations may be shared by the clonal lineages. Acute virulence, a property of all members of the type I lineage (28), is also observed in some exotic strains such as MAS and CAST, suggesting it may also be an ancestral trait. Type I strains are highly virulent in mice and may cause more aggressive ocular (23) and congenital disease in humans (34). Inheritance of acute virulence in combination with oral infectivity may have resulted in the recent expansion of strains with enhanced pathogenicity.

Our findings have important implications for the origins of foodborne zoonoses. The estimated origin of direct oral transmission in *T. gondii* is roughly concurrent with the time of human agricultural expansion (35) and adaptation of the cat as a companion animal (36), developments that created an unprecedented concentration of hosts and opportunities for new routes of transmission. Thus, changes in human behavior may have placed strong selective pressure for altered parasite transmission. Although it is generally assumed that the complex life cycles of parasites have coevolved over long time periods, our studies demonstrate that changes in transmission can occur suddenly, thereby dramatically affecting host range and pathogenesis.

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Supporting Online Material

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Figure S1
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References

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