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Evolution of Germline-Limited Sequences in Two Populations of the Ciliate *Chilodonella uncinata*

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Abstract Ciliates are microbial eukaryotes that separate their nuclear functions into a germline micronucleus and a somatic macronucleus. During development of the macronucleus the genome undergoes a series of reorganization events that includes the precise excision of intervening DNA. Here, we determine the architecture of four loci in the micronuclear and macronuclear genomes of the ciliate *Chilodonella uncinata* and compare the levels of variation in micronuclear-limited sequences to macronuclear destined sequences at two of these loci. We find that within a population, germline-limited sequences are evolving at the same rate as other putatively neutral sites, but between populations germline-limited sequences are accumulating mutations at a much faster rate than other sites. We also find evidence of macronuclear recombination and incomplete elimination of intervening DNA, which result in increased diversity in the macronuclear genome. Our results support the assertion that the unusual genomic features of ciliates can result in rapid and unpredicted patterns of diversification.

Keywords Micronucleus · Sequence evolution · Population divergence · Macronuclear recombination

Introduction

In many organisms, genomes can vary dramatically among cell types within an individual and among individuals in a population (Parfrey et al. 2008). For example, many eukaryotes differentiate their somatic genome from their germline genome by elimination and rearrangement of DNA (Zufall et al. 2005). Ciliates are microbial eukaryotes that undergo massive DNA reorganization during somatic genome development resulting in elimination of up to 98% of DNA (Prescott 1994).

Ciliates contain two nuclei in each cell: the micronucleus, which functions as the germline, and the somatic macronucleus. During vegetative growth, the micronucleus is largely inactive with most transcription originating from the macronucleus. During conjugation, meiotic products of the micronucleus are exchanged between cells, fuse, and divide mitotically, with a new macronucleus developing from at least one of the mitotic products. During development of the new macronucleus, the genome undergoes a series of rearrangements including DNA elimination and chromosomal fragmentation and amplification (Prescott 1994; Jahn and Klobutcher 2002). In some ciliates, including *Chilodonella uncinata*, the subject of this study, this process results in macronuclei with thousands of gene-sized chromosomes (Prescott 1994; Riley and Katz 2001). Such extensive genome fragmentation in ciliates is associated with the presence of highly polytenized chromosomes (Ammermann 1987), gene scrambling (Nowacki and Landweber 2009; Katz and Kovner 2010), and elevated rates of protein evolution (Zufall et al. 2006).

One class of sequence that gets eliminated during macronuclear development is internal eliminated sequences (IES), sequences that interrupt what will become the mature macronuclear chromosomes. Previous studies of variation in IES suggest a pattern of rapid divergence in IES sequence (Seegmiller et al. 1996; DuBois and Prescott 1997; Ardell et al. 2003; Huvos 2007; Mollenbeck et al. 2006; Katz and Kovner 2010). Because these sequences are

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non-coding, the evolutionary forces that would result in this rapid diversification are unclear. The presence of conserved motifs among IES in some species, including *C. uncinata*, suggests a possible role of primary sequence in regulating IES excision (Zufall and Katz 2007; Katz and Kovner 2010), however in other species, IES excision is dependent on RNA template-guided comparison of somatic and germline genomes (Mochizuki and Gorovsky 2004; Nowacki et al. 2008; Duharcourt et al. 2009).

To better understand the evolutionary forces acting on germline-limited sequences, we examined the level of sequence diversity in IES within and between geographically isolated populations of the ciliate *C. uncinata*. We compared this to variation in other genomic regions that are expected to be evolving neutrally: introns and synonymous sites in protein-coding genes. We find evidence of rapid diversification of IES between populations, but not within a population. We also find that additional sequence diversity in the macronucleus is generated by macronuclear recombination and incomplete excision of IES.

Materials and Methods

Cell Culture and Nucleic Acid Isolation

Chilodonella uncinata cell cultures from a population in the United States were obtained from the American Type Culture Collection (ATCC®50194). *C. uncinata* from a pond in Poland were obtained from S. Radzikowski (described in Robinson and Katz (2007); now deposited as ATCC®PRA-256 and PRA-257). The US and Poland cultures were both originally isolated as contaminants of other ciliate cultures (*Euplotes gracilis* and *Trithigmostoma steini*, respectively). Clonal lines were generated by passing cultures through three rounds of single-cell isolation. All strains were cultured at room temperature in bacterized Cerophyll.

Total genomic DNA was extracted from cell cultures using standard phenol/chloroform protocols (Ausubel et al. 1993). Micronuclear DNA was separated from macronuclear DNA by electrophoresis on a 0.8% (w/v) low melt agarose gel. Micronuclear DNA, which appears as a mobility-limited band on the gel, was excised and purified by digestion with AgarAce enzyme as in Zufall and Katz (2007). Total RNA was extracted with mirVana and treated with DNAse (Ambion, Austin, TX). cDNA was generated from RNA with OneStep RT-PCR kit (Qiagen, Valencia, CA).

Sequence Generation

Whole macronuclear chromosomes were cloned as described in McGrath et al. (2007) and sequenced using M13 vector primers. Primers for PCR were designed based on four new and previously published (McGrath et al. 2007) whole chromosome sequences (Table 1).

Macronuclear, micronuclear, and RNA versions of each gene were amplified from total genomic DNA, purified mobility-limited DNA, or cDNA, respectively, using iProof polymerase (Bio-Rad, Hercules, CA). PCR products were gel purified using QIAquick gel extraction kit (Qiagen, Valencia, CA) and cloned using pSTBlue-1 Perfectly Blunt Cloning kit (Novagen, Madison, WI) or Zero Blunt TOPO (Invitrogen, Carlsbad, CA). Two to five independent PCR and cloning reactions were performed for each gene from each isolate. Clones were sequenced with BigDye terminator kit (Applied Biosystems, Foster City, CA) in the Department of Microbiology and Molecular Genetics, University of Texas Health Sciences Center at Houston.

Sequence Analysis

Sequences were assembled and annotated in Geneious (Drummond et al. 2010) and were deposited in GenBank (Supplement 1). IES were identified by comparison of macronuclear and micronuclear sequences, introns by comparison of cDNA and macronuclear sequences. Polymorphisms were confirmed by the presence in two or more clones from independent PCR reactions. Resequencing was performed when necessary to confirm polymorphic sites. Sequences were aligned using Clustal W (Larkin et al. 2007; implemented in Geneious). Recombinant sequences

Table 1 Primer sequences used for amplification of each locus

Locus	Primer 1	Primer 2
PRP17[a]	F1: GCGGTCAGCATCGTAGAAGGTT	R1: AGCCGCACGTAAAGCACAGGTAG
	F2: CCAGCCCGTCGAGAGGAAGTG	R2: CTGCAAGAGGGCGGAGAGC
EIF2G	F1: TGTTGAATCCAGAAGAAGTCA	R1: TTTGTCATCTCCTCCTTTGTA
PP	F1: AATTGGTGAACGTCCTTTTAT	R1: AGCAGTTGGTACGATCCTTGGTTT
PK[a]	F1: GCGACCGGCTGGACCTCATC	R1: AGCGGGCAATCTCAACCAAT
	F2: GCTGGTGGGGGCGATGAT	R2: ACGGTTGGTGGGTTGTTTGGTC

[a] PRP17 and PK were amplified with overlapping sets of primers designated F1–R1 and F2–R2

were identified by eye. Gene genealogies of macronuclear sequences were built using PAUP* (Swofford 2003; implemented in Geneious). Nucleotide diversity was calculated in MEGA (Tamura et al. 2011). Sequence motifs in IES were identified using the MEME software suite (Bailey et al. 2009).

Results and Discussion

Micronuclear Structure

We studied four loci corresponding to four randomly sequenced full-length macronuclear chromosomes. Two of the chromosomes were previously published (McGrath et al. 2007): EF125697 has an open reading frame, the translation of which most closely BLASTs to a serine/threonine protein phosphatase (abbreviated here as "PP"); EF125698 has an open reading frame with similarity to a serine/threonine protein kinase (PK). Two of the chromosomes are newly described here: JN565724 has an open reading frame with similarity to a eukaryotic translation initiation factor 2 gamma (EIF2G); JN565722 has similarity to a pre-mRNA splicing factor 17 (PRP17).

Using primers designed from the macronuclear sequence, we determined the micronuclear structure and sequence of these loci. The structure of these loci is similar to the micronuclear structure of other *C. uncinata* loci examined, however no gene scrambling was found in any of the loci described here (Fig. 1; Zufall and Katz 2007; Katz and Kovner 2010). For two additional genes, we were unable to recover micronuclear sequence (data not shown); it is possible that this failure was due to gene scrambling. In the genes for which we obtained both micronuclear and macronuclear clones, macronuclear destined sequences (MDS) are interrupted by short IES that range in size from 30 to 187 bp (Table 2). The largest of these IES (IES4 in EIF2G) contains a microsatellite sequence (GTTTGT) that is repeated 2–17 times in the alleles sampled and is found in IES of isolates from both populations. One of the genes, PK, contains three very short (19–21 bp) introns. Short IES and rare introns now appear to be a general feature of the *C. uncinata* genome (Katz et al. 2003; Zufall and Katz 2007; Katz and Kovner 2010), similar to other ciliates with

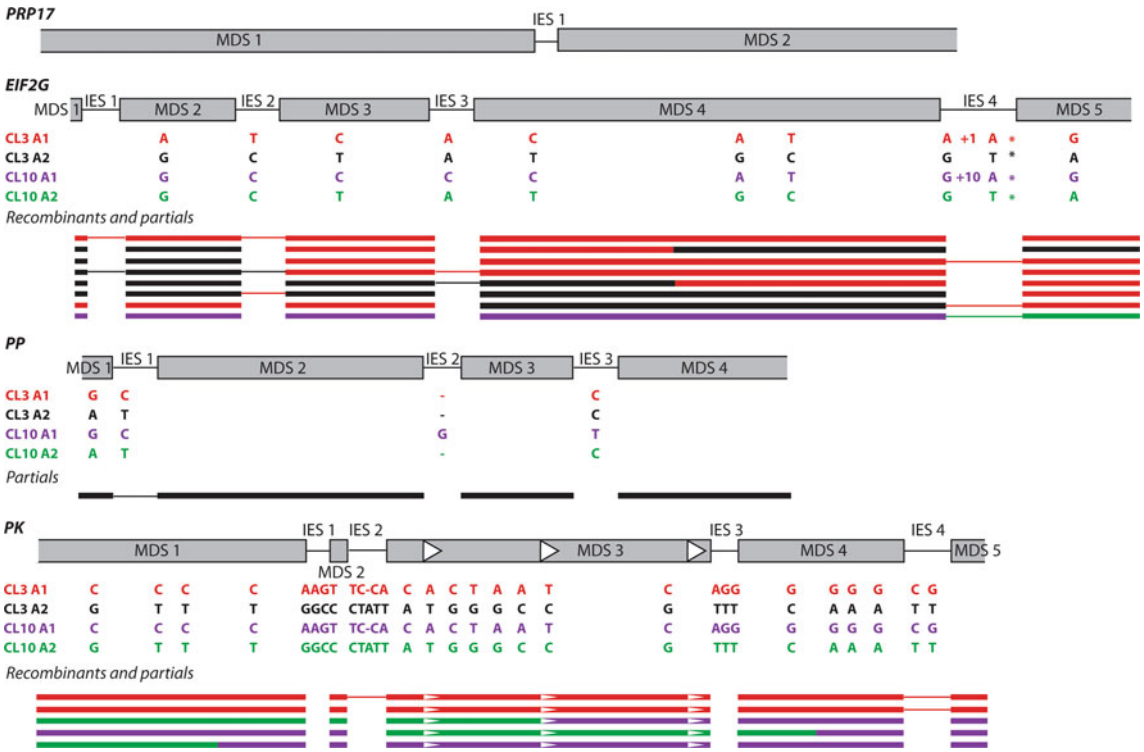

Fig. 1 Structure of four micronuclear loci in *C. unicinata*. *Gray shaded boxes* indicate sequences that are retained in the macronuclear version of the gene (MDS). MDS are interrupted in the micronucleus by IES. *White triangles* in PK MDS3 indicate the location, and approximate size, of introns. Beneath EIF2G, PP, and PK are shown the polymorphic sites in the US alleles and the structure of clones that were recombinants between alleles and/or contained some, but not all IES ("partial" excision). Colors on the recombinant and partial clones indicate sequence corresponding to the allele that is shown in that color. In EIF2G IES4, +1 and +10 indicate an additional one or ten repeats of the sequence GTTTGT in the A1 alleles relative to the A2 alleles. * represents nine polymorphic sites at this location that are identical in all sequences except CL10 A1 (see Supplement 2)

Table 2 IES lengths and pointer sequences for each locus from the two populations

Locus	Population	IES1[a]	IES2	IES3	IES4
PRP17	US	(30) TTCTTGG			
EIF2G	US	(66) CTTGAACC	(73) CAATATCAC	(72) GGCTGCATTG	(125-183)[b] TGTAAGGT
	Poland	(59) CTTGAACC	(50) CAATATCAC	(66) TGCATTG	(69) GGTAAGGTC
PP	US	(71) AAGATCC	(64–65)[b] TGGACT	(74) GGTCTTGA	
	Poland	(74) AAGATCC	(65) GGACTTT	(77) CTTGAG	
PK	US	(42) AGTCAGAAT	(59–60)[b] GATACATCCT	(50) AGATGGG	(79) CCCAGC

[a] In parentheses are the lengths of each IES (nucleotides). The sequence is the pointer sequence for that IES. The pointer sequence is a direct repeat at the beginning and end of each IES; one copy is retained in the MDS, the other is eliminated during IES excision

[b] Indicates variation in IES length within a population due to indels or microsatellite variation (EIF2G IES4)

extensively fragmented genomes (e.g., DuBois and Prescott 1997; Hogan et al. 2001; Chang et al. 2006).

Sequence Diversity Within and Between Populations

We amplified micronuclear and macronuclear DNA for three loci from four single-cell isolate clonal lines, two from a population in the US (CL3 and CL10) and two from a population in Poland (CLG and CLB). One locus, PRP17, could not be consistently amplified from any of the cell lines except CL3, thus, we did not include it in the population analyses. Another locus, PK, could only be amplified from the US isolates, despite attempted amplification with multiple primer pairs and PCR conditions, suggesting that it is either highly divergent or absent from the Poland isolates.

The US isolates are heterozygous at all loci examined and sequences vary between cell lines, indicating the presence of greater than 2–4 alleles in this population. Further, the micronuclear and macronuclear alleles are identical (with the exception of recombination, see below) at MDS, suggesting that the observed variation is unlikely due to mutations occurring during asexual division in culture. Despite being derived from a stock center culture, which are often assumed to represent a single clone, this sequence variation, in combination with the ability to amplify PRP17 from only one of two isolates indicates that this culture contains multiple independent cell lines. Thus, we feel confident using these isolates to study within population variation. The Poland isolates are completely homozygous and cell lines are identical, suggesting that the original culture was likely derived from a single inbred individual.

Genealogies were constructed using macronuclear sequences because IES are too divergent to align (see below). EIF2G and PP demonstrate the deep divergence between the US and Poland populations, with low levels of allelic diversity within the US population relative to between the US and Poland populations (Fig. 2), suggesting a long time of separation with little to no gene flow between these populations.

Within the US population, we compared levels of sequence diversity in IES to synonymous sites and introns,

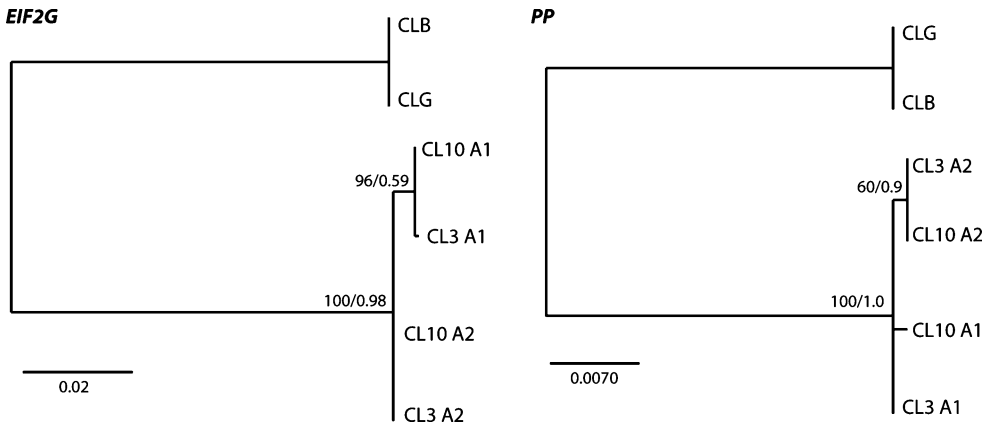

Fig. 2 Genealogies of alleles from two macronuclear loci. Alleles are designated by the clone line from which they were derived (CL). CL3 and CL10 are from the US population; CLG and CLB are from the Poland population. In the heterozygous clone lines, alleles are named A1 and A2. Topologies and branch lengths are derived from maximum likelihood analyses of aligned sequences. Support at nodes is from 100 maximum likelihood bootstrap replicates (calculated in PAUP* (Swofford 2003)) and the bayesian posterior probability (calculated in MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003)). Scale bars show substitutions per site

which are presumably evolving neutrally. We found no significant differences in levels of diversity among these regions within each gene (Table 3). However, between the US and Poland populations, while the MDS align unambiguously and 3rd codon positions do not appear to be saturated (EIF2G mean diversity between populations at synonymous sites (dS) = 0.20; PP dS = 0.12), all of the IES are too divergent to align between populations (for examples, see Fig. 3). The position of IES excision relative to the coding region is identical between populations. The pointer sequences, i.e., direct repeats that bound IES, are nearly identical, with the exception of evidence of MDS-IES junction shifts (DuBois and Prescott 1997; Wong and Landweber 2006) in three IES (Table 2; Fig. 3). These results demonstrate the rapid diversification of IES between populations relative to MDS, and relative to the level of IES sequence diversity found within populations. These results are consistent with previous studies of between population variation in this and other ciliates (Katz and Kovner 2010; Mollenbeck et al. 2006) and suggest that IES sequence divergence occurs between populations more rapidly than changes in IES number and position, which is often found between species (DuBois and Prescott 1997; Huvos 2004; 2007; Mollenbeck et al. 2006).

Table 3 Nucleotide diversity in various gene regions between alleles from the US population

Locus	Synonymous sites[a]	Introns[a]	IES[a]
EIF2G	0.014 (0.005)	N/A	0.006 (0.003)
PP	0.003 (0.003)	N/A	0.006 (0.004)
PK	0.024 (0.007)	0.023 (0.017)	0.041 (0.012)

[a] Nucleotide diversity is calculated in MEGA (Tamura et al. 2011) as the overall mean distance between alleles, using the Jukes–Cantor correction. Standard error, shown in parentheses, is estimated by 500 bootstrap replicates. There is no significant difference in diversity between gene regions within any loci (t-test, $P > 0.05$)

Conserved IES Motif

We compared sequences across all of the IES to assess the presence of conserved motifs, which have previously been found in *C. uncinata* (Katz et al. 2003; Zufall and Katz 2007; Katz and Kovner 2010). We found a conserved motif that is consistent with previous reports. It is present in all IES from both populations: TGWTTDTRKK (E-value = 9.6; E-value of motif found with scrambled sequences = 5.0×10^6). Interestingly, this motif contains the microsatellite sequence found in IES4 of EIF2G (see above). We repeated the analyses with this IES and recover a nearly identical consensus motif: TGATTRTDTK. It is possible that the overrepresentation of this sequence in IES allowed for its expansion into a microsatellite. While the precise role of this motif in IES excision is unclear, the fact that it is consistently found in nearly all *C. uncinata* IES examined to date (Katz et al. 2003; Zufall and Katz 2007; Katz and Kovner 2010) strongly suggests that it plays an important *cis*-acting role in developmental sequence elimination. In other ciliates, IES excision is primarily determined by an RNA-mediated epigenetic process that is apparently not dependent on *cis*-acting sequences (Mochizuki and Gorovsky 2004; Nowacki et al. 2008; Duharcourt et al. 2009); whether or not such a mechanism exists in *C. uncinata* is unknown.

Macronuclear Recombination and Incomplete IES Excision

Multiple macronuclear recombinants were detected among alleles in a cell line for some loci (Fig. 1; Table 4). No

Fig. 3 Examples of sequences of IES and surrounding MDS from the US and Poland alleles demonstrate similarity in MDS and divergence in IES. The top sequence in each alignment is an allele from the US population; the bottom sequence from the Poland population. The IES sequence is boxed; MDS sequence is outside of the box. Pointer sequences are in bold. PP IES2 demonstrates a case of an MDS–IES junction shift (DuBois and Prescott 1997)

Table 4 Number of clones of each allele sequenced

Locus	Allele[a]	MIC[b]	MAC[c]	Partial[d]
PRP17	CL3 A1	6	7	N/A[e]
EIF2G	CL3 A1	2	2	2
	CL3 A2	4	5	0
	CL3 R	0	1	5
	CL10 A1	2	4	0
	CL10 A2	9	4	0
	CL10 R	0	0	1
	CLG	7	6	0
	CLB	7	8	0
PP	CL3 A1	3	3	0
	CL3 A2	5	13	1
	CL10 A1	3	2	0
	CL10 A2	7	32	0
	CLG	8	5	0
	CLB	8	7	0
PK	CL3 A1	10	4	0
	CL3 A2	13	1	2
	CL3 R	0	0	0
	CL10 A1	2	2	0
	CL10 A2	5	1	0
	CL10 R	0	3	0

[a] Alleles are named by clone line (CL) and numbered A1 or A2, as in Fig. 2

[b] Micronuclear (MIC) clones are determined by the presence of all IES in a single clone

[c] Macronuclear (MAC) clones are those with no IES

[d] Partial clones have some, but not all, IES

[e] PRP17 contains only one IES, so partial clones would not be detected

Alleles designated 'R' are recombinants between alleles A1 and A2

Sequences of recombinant and partial clones are in Supplement 2

micronuclear sequences (i.e. sequences with all IES intact) showed any evidence of recombination, indicating that the observed recombination was unlikely an artifact of PCR or cloning. Approximately 25% of macronuclear sequences from EIF2G and PK (including those with partial IES excision, see below) appear to be recombinants (Fig. 1; Table 4). This is similar to the frequency of macronuclear recombination found in *Tetrahymena* (Deak and Doerder 1998). No recombinants could be detected in PP or in the Poland cell lines due to the lack of variation within an isolate.

Several clones were sequenced that contained some, but not all of the IES identified in the micronuclear sequences. In particular, $\sim$1/3 of all EIF2G clones with any missing IES, i.e., presumed macronuclear clones, had only some of the IES excised (Table 4). In this gene, partial IES excision is significantly associated with recombination (Fisher's exact test, $P < 0.01$; Fig. 1). Fewer incompletely processed clones were found for PP (1/51 macronuclear sequences) and PK (2/13). Incomplete IES excision could be a result of either the presence of developmental intermediates or inefficient excision of some IES (Jahn and Klobutcher 2002). No incompletely processed clones were found in the Poland cell lines.

Given the fact that these cell lines had not recently undergone conjugation, we conclude that at least in the US cell line, IES can experience inefficiency in IES excision, and this inefficiency is more pronounced in the presence of divergent alleles. If allelic diversity does result in improper excision of IES, we may expect to find selection acting to reduce allelic diversity among interbreeding populations of ciliates. This may also help to explain our observed pattern of IES divergence: within a population, high levels of IES diversity are not tolerated because of the resulting improper excision, but between populations that do not frequently produce heterozygous progeny, IES are free to diverge.

Conclusion

In this study, we characterized the micronuclear and macronuclear structure of four loci in a ciliate that undergoes extensive developmental genome fragmentation. By comparing sequence variation in two of these loci within and between populations of *C. uncinata*, we found rapid divergence of germline-limited sequences between populations, but no higher than expected levels of within population diversity in comparison to other neutral sites were found. This rapid diversification between geographically isolated populations and between species now appears to be a general feature of IES evolution. In addition, we describe multiple mechanisms in *C. uncinata* that can generate diversity in macronuclear genome content: sequence evolution, macronuclear recombination, and incomplete processing of macronuclear chromosomes. Although not found among the loci studied here, alternative processing of scrambled genes (Katz and Kovner 2010) can additionally add to this diversity. These results confirm previous studies that suggest that the genome architecture, cell biology, and life history of ciliates can result in unpredicted, and unusually rapid, patterns of evolution (e.g., DuBois and Prescott 1997; Wong and Landweber 2006; McGrath et al. 2006; Zufall et al. 2006; Huvos 2007; Katz and Kovner 2010).

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