Frequent Coinfection Reduces RNA Virus Population Genetic Diversity

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Abstract

The masking of deleterious mutations by complementation and the reassortment of virus segments (virus sex) are expected to increase population genetic diversity among coinfecting viruses. Conversely, clonally reproducing or noncoinfecting virus populations may experience clonal interference where viral clones compete with one another, preventing selective sweeps. This dynamic reduces the efficiency of selection and increases the genetic diversity. To determine the relative influences of these forces on population genetic diversity, we evolved 6 populations of bacteriophage φ6 under conditions promoting or preventing coinfection. Following 300 generations, we isolated and partially sequenced 10 clones from each population. We found greater diversity among asexual populations than sexual populations. Moreover, sexual populations did not show greater relative fitnesses than asexual populations, implying that reduced genetic variation did not result from purifying selection. However, sexual populations were less genetically robust than asexual populations and likely more prone to the deleterious epistatic effects of mutations. As such, a neutral mutation on the asexually evolved (robust) background could be profoundly deleterious on the sexually evolved (brittle) background. This could facilitate sexual populations undergoing greater purifying selection to remove deleterious mutations, but this selection is not reflected by increases in average population fitness. Our results bolster a growing literature suggesting that RNA virus segmentation is probably not a mechanism that evolved because it provides a generalized benefit of sex.

Key words: bacteriophage, clonal interference, complementation, evolution of sex, nucleotide diversity, segmented virus

Genetic diversity—the raw material for evolution via natural selection—is an essential factor in determining whether organisms can adapt to novel or changing environments. RNA viruses are believed to have considerable evolutionary potential because of their high genetic diversity. The ultimate source of this genetic diversity is clear. Because their genomes are copied using RNA-dependent RNA polymerases that have no proofreading activity, RNA viruses possess among the highest mutation rates of any biological entities (Drake 1993; Drake and Holland 1999; Duffy et al. 2008; García-Villada and Drake 2012). However, it is less clear how virus reproduction mode (sexual vs. asexual) affects the maintenance of standing genetic diversity in RNA virus populations.

Virus sex (recombination, reassortment) can only occur when multiple virus genotypes coinfect the same host cell. Competing hypotheses predict different effects on standing genetic diversity in virus populations experiencing frequent versus infrequent opportunities for genetic exchange (sex). On one hand, frequent virus coinfection may inflate population genetic diversity. During coinfection, virus genetic material can be recombined (or reassorted) to produce genetically distinct offspring (Chao 1992). In addition, coinfecting viruses can experience complementation where viruses defective or disadvantaged in the production of essential proteins can evade selection by co-opting functional or superior proteins produced by conspecifics. In such cases, viruses possessing multiple deleterious mutations can persist, elevating population genetic diversity (Szathmáry 1992; Manrubia and Lazaro 2006). Collectively, these processes may increase genetic diversity and capacity for evolutionary change among viruses experiencing frequent coinfection.
On the other hand, RNA virus populations experiencing frequent asexual (clonal) reproduction may show more genetic diversity than those experiencing frequent coinfection. In clonal populations of large size, multiple genotypes of approximately equal fitness can coexist for long periods because selection is ineffective at discriminating between them, an effect known as clonal interference (Fisher 1930; Muller 1932; Hill and Robertson 1968; Gerrish and Lenski 1998; Kim and Orr 2005; Bollback and Huelsenbeck 2007; Pepin and Wichman 2008; Miller et al. 2011; Rokyta et al. 2011; Hughes et al. 2012; Keller et al. 2012; Raynes et al. 2012). Moreover, in sexual populations, linkage disequilibrium will purge diversity in genomic regions adjacent to beneficial alleles as selected sites sweep to fixation. Therefore, the weaker efficiency of selection on beneficial alleles in rarely coinfecting populations can cause standing genetic diversity to be greater, relative to populations where coinfection is common.

To summarize, virus sex is expected to increase genetic diversity because 1) complementation can mask effects of lethal or deleterious mutations from selection and 2) recombination can increase the number of haplotypes in a population. By contrast, clonally infecting populations may experience higher genetic diversity because of clonal interference, which reduces the effectiveness of selection in purging genetic diversity.

Experimentally measuring genetic diversity within coinfecting and clonally infecting populations may shed light on the consequences of coinfection for RNA virus populations. Here, we examined sequence diversity of replicate populations of the tripartite double-stranded RNA phage φ6 experimentally evolved under high versus low levels of coinfection. The frequency of coinfection was experimentally manipulated by controlling the ratio of phages to bacteria, often referred to as the multiplicity of infection (MOI). At high MOIs, phages outnumber bacteria, and many hosts adsorb more than 1 phage and are coinfected. In phage φ6, this process allows formation of hybrid reassortants: progeny viruses containing a mixture of the 3 genomic segments present in the multiple phages that coinfected the cell (Turner and Chao 1998; Turner et al. 1999). Thus, from populations experimentally evolved under high and low MOIs, we can determine the relative influences of coinfection on genetic diversity.

Materials and Methods

Strains and Culture Conditions

Phage φ6 (family Cystoviridae) is a lytic virus with a 13 388 bp double-stranded RNA genome divided into small (2948 bp), medium (4061 bp), and large (6374 bp) segments (Mindich 2006). All strains were derived from a clone of wild-type φ6 and were grown on the typical laboratory host, Pseudomonas syringae pathovar phaseolicola HB101Y (ATCC #21781). Pseudomonas syringae pathovar phaseolicola was grown in 10 mL lysogeny broth (LB) medium (10 g Bacto™ tryptone, 5 g Bacto™ yeast extract, and 10 g of NaCl per liter double-distilled H2O, pH 7.5) in a 25 °C incubator with rotary shaking (120 r.p.m.). Phage stocks were prepared by mixing viruses and stationary-phase (24 h old) P. phaseolicola in 3 mL of 0.7% LB top agar overlaid onto 1.5% LB agar. After overnight incubation at 25 °C, phages formed visible holes (plaques) in the bacterial lawn. Virus lysates were prepared by harvesting and resuspending plaques in 4 mL LB broth, followed by centrifugation and filtration (0.22 μm, Durapore™; Millipore, Bedford, MA) of supernatant to remove cells. Virus lysates and single-cloned isolates were stored for up to 5 days at 4 °C or permanently at −20 °C in a 4:6 glycerol/LB (v/v) solution.

Experimental Evolution

Previously, an evolution experiment was performed where a single clone (plaque) of wild-type φ6 was used to found 3 populations in each of 2 treatments: low initial ratio of phages to bacteria (populations L1, L2, L3) and high initial ratio of phages to bacteria (populations H1, H2, H3) (Turner and Chao 1998). Phages and bacteria were mixed in LB broth for 40 min at 25 °C to allow virus adsorption to cells; L lineages were mixed at an MOI of 0.002 (4 × 106 phages to 2 × 107 cells); H lineages at an MOI of 5 (1 × 1010 phages to 2 × 107 cells). A diluted sample of the mixture containing “infec tive centers” (infected cells) was then plated on a P. phaseolicola lawn to yield ~500 plaques per plate. The overwhelming majority of plaques in the high-coinfection treatment were founded by multiple phages in a single-infective center, whereas those in the low-coinfection treatment were founded by only a single phage, on average. After 24 h, each infective center yielded a single plaque formed through approximately 5 generations of virus infection and lysis of neighboring cells in the lawn. Plaques containing virus progeny were harvested and filtered to obtain a cell-free lysate, and the process was repeated. The populations were experimentally evolved for 300 generations (60 passages) totally: 250 generations in the original study (Turner and Chao 1998) followed by an additional 50 generations under identical culture conditions (Montville et al. 2005). Figure 1 shows a schematic of the experimental design. We note that the opportunity exists for genetic exchange between phage genotypes within plaques as they form on agar, emphasizing that both treatments allowed some degree of viral sex; thus, the manipulation of MOI that preceded plating minimally defined the sexual versus asexual difference between treatments.

Sequencing

Following 60 passages (generation 300), 10 single clones were isolated by randomly choosing 10 individual plaques from each experimental population (60 clones total), and these were subjected to partial genome sequencing. In laboratory experiments with some viruses, defective genotypes (deletion mutants) can be enriched when populations are maintained at high MOIs. These genotypes are unable to reproduce on their own and require the presence of helper viruses to produce requisite proteins; if these mutants reduce fitness of coinfected full-length helpers, they are...
termed defective-interfering particles (Holland 1991). If present, defective phage could possibly skew our estimates of population genetic diversity because they are unable to form plaques in the absence of helper phage. Any defective phage (and the genetic diversity they contribute to the population) would be lost when plating for single clones. However, defective phages have never been observed in φ6 experiments at high MOI (Turner and Chao 1998; Turner and Chao 2003). Rather, full-length “cheater” phage can evolve when phage φ6 is cultured at high MOI (Turner and Chao 1999). Plating single clones from 60th passage lysates would effectively sample these phages in proportion to their presence in the parent population. Therefore, the available literature shows no evidence that our strategy of isolating 10 single clones should bias against random isolation of genotypes in the treatment populations.

To obtain partial genome sequences from a phage clone, 3 mL of a high titer lysate (~10^10 phages per milliliter) of the clone was ultracentrifuged (107,000 r.c.f.) to concentrate phage particles. Following centrifugation, supernatants were removed, and pellets were resuspended in 150 μL RNase-free water. Purified RNA was extracted from concentrated samples using a QIAamp® Viral RNA Mini Kit (Qiagen, Valencia, CA). Phage RNA was converted into complementary DNA using SuperScript® II Reverse Transcriptase and random hexamer primers (Life Technologies, Grand Island, NY).

We examined molecular evolution at loci on the small and large segments, which should experience different selection pressures. Primer pairs S7/S8 (5′ TGA GGAGGTTCCGGCCAAC and 5′ GACTGACGAC GGGGAGTAGATTTC) and L9/L22 (5′ CTTCAAAGAGGGGCGATTG and 5′ ACGCCGCTCGCAGAA AAAAC) were used to amplify ~700 bp regions overlapping both coding and noncoding regions of the genome. L9/L22 amplified a portion of the large segment that codes for the highly conserved RNA-dependent RNA polymerase, protein P2 (Bruenn 1993). The region on the small segment amplified by S7/S8 contains the C-terminal end of the cell-wall lysis protein, but the majority of this region is not protein coding (Mindich 2006). In addition to being mostly noncoding RNA, the sequenced coding portion of the small segment might be under weaker selection because it is the least translated portion of the genome.
q6 genome (Sinclair et al. 1975)—it has an alternate start codon and is the furthest gene from a ribosome binding site (McGraw et al. 1986).

Polymerase chain reaction (PCR) amplifications were performed using standard methods with High Fidelity Platinum® Taq DNA polymerase (Life Technologies). PCR products were purified for sequencing using ExoSAP-It (Affymetrix, Santa Clara, CA). Sequencing was performed using BigDye reagents (Applied Biosystems, Foster City, CA) on an ABI 3100 sequencer (Applied Biosystems).

Sequence Analysis

Sequences were edited using Sequencer 4.7 (Gene Codes Corporation, Ann Arbor, MI) and aligned using ClustalX (http://www.clustal.org/clustal2/). The small-segment and large-segment partial sequences were trimmed to a length where all 60 clones had coverage with at least 2 independent sequencing reactions (658bp on the small segment, 665bp on the large segment, totaling ~10% of the genome). LAMARC v2.1.3 (http://evolution.genetics.washington.edu/lamarc/index.html) was used to estimate the parameter $\theta_{\text{MLE}}$ using a maximum likelihood approach based on coalescent theory (Beerli and Felsenstein 2001; Kuhner 2006). $\theta_{\text{MLE}}$ was estimated using a Metropolis-Hastings Monte Carlo method, which samples among possible coalescent trees describing the relationship among the sampled sequences according to their likelihood under a given model of sequence evolution (Kuhner et al. 1995). For these analyses, clonal sequences obtained from small and large segments were denoted distinct genetic regions and combined into 1 sequence, resulting in a 2-locus estimate of $\theta_{\text{MLE}}$. All analyses used the F84 model of sequence evolution (Felsenstein 1984) with default values assigned for base frequencies and transition/transversion ratios. No migration was assumed. Because the Metropolis-Hastings sampling method is starting point dependent, we conducted 5 replicate analyses for each population, and the results were compared. Runs were performed using the default search strategy: 10 short chains with 500 trees sampled and 2 long chains with 10 000 trees sampled (1000 trees discarded as burn-in).

Fitness Assays

Phage fitness was determined using paired-growth assays, following published methods (Dennehy and Turner 2004). The fitness assay compared 24-h growth on P. phaseolicola of a focal strain relative to a common competitor carrying a genetic marker (i.e., ability to infect Pseudomonas phaseolicola bacteria). The competitors were mixed in a 1:1 ratio, and then a dilution of this mixture containing ~400 viruses was plated on a P. phaseolicola lawn. Because no pre-attachment occurred before plating, every virus in the lawn infected a cell alone. After 24-h incubation, ~400 plaques were harvested and filtered to obtain a cell-free lysate. The ratios of competing genotypes in the starting mixture ($R_0$) and in the harvested lysate ($R_t$) were obtained by plating on mixed lawns of P. phaseolicola and P. pseudalcaligenes (200:1 mixture), where ordinary and host-range (here, common competitor) genotypes form turbid and clear plaques, respectively. Fitness ($W$) was defined as the relative change in the ratios of ordinary to common competitor virus, or $W = R_t/R_0$.

Results

Within- and between-Population Genetic Diversity

Two genomic regions were sequenced from each of 60 clones, 10 clones from each of the 3 sexually and 3 asexually evolved populations (Montville et al. 2005). The observed mutations are shown in Figure 2. Eight different mutations were found in sequenced protein-coding regions, 5 of which were synonymous mutations. Neither of the nonsynonymous mutations was observed in more than 1 population, preventing any inference of fitness benefits of these mutations due to convergence. However, 2 of the synonymous mutations appeared to be beneficial in the selective environment: generalized growth on P. phaseolicola. The synonymous mutation P197P in gene P5 of the small segment was present in some sequenced clones from populations L1 and L2, and appeared to have fixed in lineage H3. The synonymous mutation F169F was also observed in a subset of clones drawn from populations L2 and L3. These synonymous changes to the lysis protein appear to beneficially alter q6 translation kinetics regardless of the frequency of coinfection. Such situations can result in codon bias (reviewed by Hershberg and Petrov 2008).

Many mutations were also observed in the sequenced noncoding region of the small segment, but no single mutation was present in more than 1 population (Figure 2). Most importantly, all of these mutations in the noncoding region were found exclusively in the asexually evolved populations. These 8 noncoding mutations account for the difference in the number of mutations observed between the 2 treatments (13 unique mutations in the asexually evolved populations, 4 in the sexually evolved populations). Populations experiencing the high MOI treatment had significantly fewer mutations than populations subjected to the low MOI treatment (analysis of variance [Anova]: $F = 3.903$, degrees of freedom [df] = 1, $P < 0.05$).

We observed from 1 to 6 haplotypes within each evolved population: L1, 6; L2, 6; L3, 3; H1, 3; H2, 1; H3, 2. The effect of treatment on observed number of haplotypes approached statistical significance (unpaired $t$-test: $t = -2.530$, df = 4, $P = 0.0647$). The greater clonal diversity of the asexual populations is also reflected in the average nucleotide diversities $\theta$ over all loci of the populations (Figure 3). An unpaired $t$-test of these results by coinfection treatment approached statistical significance ($t = 2.449$, df = 4, $P = 0.0705$). We concluded that the asexually evolved q6 populations showed slightly more genetic diversity than the sexually evolved populations, on average, based on sequence data representing ~10% of the viral genome.

No Fitness Effects of Sequenced Diversity

One explanation for the lower genetic diversity observed in the sexually evolved lineages is that they experienced more
diversity-reducing selective sweeps and purifying selection than their sexual counterparts (this is obviously the case with population H3). This potential difference might cause the sexually evolved populations to have higher fitnesses due to fixation of beneficial mutations. Alternatively, the asexually evolved populations might show lower fitness values and higher genetic diversity because of larger numbers of accumulated mildly deleterious mutations. To examine whether the MOI treatments led to measurable fitness differences between viruses, we analyzed the fitness data of all 60 clones originally presented in our earlier study (Montville et al. 2005). We observed that the fitnesses of the clones did not statistically differ according to coinfection treatment (Figure 4; Anova: \( F = 0.05, P = 0.84 \)). This result strongly suggests that the more diverse asexually evolved clones did not experience greater numbers of beneficial or deleterious mutations relative to the sexually evolved clones. We conclude that the diversity observed in the 2 sequenced regions did not lead to fitness differences among the 60 clones.

### Discussion

**Greater Genetic Diversity in Asexual Populations**

Sexual populations are often more genetically diverse than asexual (clonal) ones (Simon et al. 1996; Colegrave 2002; Delmote et al. 2002; Chapman et al. 2003; Jokela et al. 2003; Grapputo et al. 2005; Olarte et al. 2012). Some studies allow for the possibility that increased genetic variability can foster faster adaptation (Rice and Chippindale 2001; Colegrave 2002; Colegrave et al. 2002; Kaltz and Bell 2002; Goddard et al. 2005; Renaut et al. 2006). However, we observed that high MOI (sexually evolved) populations of phage \( \phi 6 \) were less genetically diverse (i.e., fewer total mutations and inferred haplotypes) than their low MOI (asexually evolved)
counterparts following 300 generations of experimental evolution. Although both treatment groups showed similar
diversity in protein-coding regions, the asexually evolved
populations were more diverse in noncoding regions (7
mutations vs. none, see Figure 2). These results appear to
contradict theory that predicts greater genetic diversity in
coinfecting virus populations relative to clonal ones (Frank
2001) and suggest that the reassortment (sexual exchange)
of genomic segments may not generate increased genetic
variation that would foster faster adaptation in these RNA
viruses (Chao 1988; Chao 1992). Even if our conclusions
are in error, and there are no significant differences in the
genetic diversities of the 2 treatments, this result still is at
odds with the theoretical expectation that viral populations
experiencing high levels of coinfection should show significa-
cantly greater standing genetic variation than those evolved
under clonal conditions.

Following 300 generations of viral evolution, the mean
level of nucleotide diversity in our experimental populations
was almost an order of magnitude lower than those reported
among RNA virus populations infecting humans (Elena,
Codoner, and Sanjuan 2003) and within experimentally

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**Figure 3.** Population average nucleotide diversity (θ). θ was computed using a maximum likelihood approach. Each value of θ is the average of 5 estimates, enabling the computation of a 95% confidence interval.

**Figure 4.** Relative fitness estimates for clones obtained from asexually and sexually evolved populations. Ten clones were isolated from each of 3 low MOI (L1, L2, L3) and high MOI (H1, H2, H3) phage φ6 populations. Each point is the mean of 3 relative fitness estimates for each clone. Global mean standard error < 0.013. Some points offset for clarity.
evolved populations of RNA phage Qβ (θ = 0.0017, 0.0018; these estimates derived from our analysis of Bacher et al. 2003. One explanation is that the mutation rate of phage φ6 (estimated to be $2.7 \times 10^{-6}$ reversions per generation; Chao et al. 2002; Burch et al. 2007; Ferris et al. 2007) is generally lower than that of most RNA viruses (Drake 1993; Drake and Holland 1999). Although the sexually evolved populations were especially lacking in genetic diversity, this dearth of mutations did not appear to be due to a lower relative mutation rate. We previously observed that the sexually evolved viruses had a greater tendency to generate spontaneous host-range mutants when challenged to grow on a novel host species (Montville et al. 2005), suggesting they are not mutation limited. However, this difference in ability to generate host-range mutants was most likely due to differing genetic architectures between the asexually and sexually evolved strains (e.g., presence of preexisting mutations in the sexually evolved strains, which were required for a 2-step mutation process to infect the new host). Thus, the available evidence does not indicate that differences in mutation rate can explain our current results.

**Clonal Interference**

One possible explanation is that clonal interference caused genetic diversity to be higher in the asexually evolved populations, on average, compared with the sexually evolved populations. Recombination (breaking and joining of homologous sequences) is rare or nonexistent in phage φ6, but reassortment of segments readily occurs. Populations of the virus evolved under low MOI were not allowed to experience frequent reassortment, thus preventing these lineages from combining multiple beneficial mutations that arose in different genetic backgrounds. A consequence was that mutants of equal (or very similar) fitnesses might have been maintained together in the population for longer times due to inefficiency of selection. The relatively greater number of inferred haplotypes in the asexually evolved populations was consistent with the hypothesis that clonal interference maintained neutral genetic variation in these populations but not in the sexually evolved lineages. This hypothesis is further supported by our observations that clone fitness was not highly divergent among viruses within each asexually evolved population and that much of the diversity was found in noncoding regions. However, we note that other processes, such as purifying selection, can produce the same results.

We note that the likelihood of clonal interference should increase with elevated population size (Miralles et al. 2000). Although in the original experimental evolution study an attempt was made to create equivalent effective population sizes (i.e., ~500 individuals) across the high and low MOI treatments (Turner and Chao 1998), it was later shown that a limit to coinfection in phage φ6 probably caused the effective population size at high MOI to be between 200 and 300 individuals (Turner et al. 1999). These effective population sizes were thus similar, but the difference might have enhanced the opportunity for clonal interference to operate under low MOI conditions.

The high MOI populations, by contrast, could have experienced reassortment that combined multiple beneficial mutations (i.e., mutated segments) into the same background, allowing more efficient fixation and reduced overall population genetic diversity (Fisher 1930; Muller 1932). Such a scenario would be consistent with the Fisher–Muller hypothesis, which states that recombination will increase the rate of adaptation. However, we found no evidence that the sexually evolved populations showed higher average fitness than their asexually evolved counterparts; rather the within-population fitness diversity was roughly equal between the 2 treatments. This result implied that the sexually evolved populations did not experience greater fitness gains and did not have lower genetic variance in fitness than the asexually evolved populations.

**Mutational Robustness**

Our results are also consistent with the reported difference in evolved genetic robustness (phenotypic constancy in the face of mutational change) among the φ6 populations. We previously found that when mutation accumulation was used to randomly introduce mutations into the virus populations, the sexually evolved lineages were less robust than the asexually evolved lineages (Montville et al. 2005). By definition, robust populations should show greater diversity than brittle ones because robustness essentially creates greater neutrality among variants within a population. Therefore, the greater number of inferred haplotypes in the asexually evolved populations is expected based on earlier robustness results with these evolved populations. One possibility for future study would be to use next-generation sequencing to explore rare diversity in the evolved populations and to identify whether the purported effects of clonal interference and/or differing evolved robustness caused the asexually evolved populations to retain variation that might be beneficial in new environments, possibly enhancing their evolvability.

A test of the advantage of sex using this same study system showed somewhat different results; low MOI asexual and high MOI sexual populations of φ6 were compared for their abilities to purge mutations of known deleterious effect (Froissart et al. 2004). The results indicated that standing genetic diversity existed for longer in the sexually evolved populations, evidenced by deleterious mutations purged significantly faster in absence of coinfection (asexual conditions). The explanation was frequent coinfection resulted in complementation, which buffered the effects of deleterious mutations and caused selection to be inefficient at purging them. However, there was 1 key difference between that previous study and the present one. In the study by Froissart et al. (2004), only the fate of deleterious mutations was examined, whereas in this present study, all mutations in the sequenced regions, including beneficial and neutral mutations, were considered. It may be that discrepancy in results between the 2 studies can be explained by clonal interference. Competing beneficial mutations of similar effect may be maintained longer in asexual populations, increasing population genetic diversity.
Virus Segmentation Not for Sex

Taken together, the results show that sexually evolved populations of phage φ6 do not maintain higher levels of genetic diversity (this study), do not adapt faster to a fixed environment (Turner and Chao 1998), and do not purge deleterious mutations faster via reassortment (Froissart et al. 2004). Additionally, reassortment among φ6 phages facilitates complementation, and deleterious alleles, cheating genes, and defective segments are maintained longer than they are in asexually evolved populations (Turner and Chao 1998; Turner and Chao 1999; Froissart et al. 2004). Although evidence shows that reassortment allows φ6 populations to recover from the effects of accumulated mutations due to Muller's ratchet (Chao et al. 1992), these combined results indicate that sex is not generally beneficial in evolving populations of the virus and that clonal interference is apparently a stronger force than complementation for maintenance of standing genetic diversity.

The evolutionary ecology of RNA virus segmentation has been perhaps studied more intensely in phage φ6 than in any other segmented virus. In the broadest sense, it appears that genetic exchange in φ6 may be an occasionally useful side effect of a segmented genetic architecture, which evolved for another purpose (Pressing and Rannney 1984; Nee 1989). A likely possibility is that segmentation evolved to promote greater packaging efficiency of RNA and better control of gene expression in the virus (Onodera et al. 1998; Qiao et al. 2000). This possibility echoes the important difference between the evolutionary origins of sex versus its maintenance, a difference that is often discussed in the evolution of sex literature. Although segmentation in RNA viruses might have evolved for a different purpose, reassortment may be occasionally useful and evolutionarily important in these viruses even if it occurs rarely.

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