The Cobweb of Life Revealed by Genome-Scale Estimates of Horizontal Gene Transfer

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With the availability of increasing amounts of genomic sequences, it is becoming clear that genomes experience horizontal transfer and incorporation of genetic information. However, to what extent such horizontal gene transfer (HGT) affects the core historical genealogy of organisms remains controversial. Based on initial analyses of complete genomic sequences, HGT has been suggested to be so widespread that it might be the “essence of phylogeny” and might leave the treelike form of genealogy in doubt. On the other hand, possible biased estimation of HGT extent and the findings of coherent phylogenetic patterns indicate that phylogeny of life is well represented by tree graphs. Here, we reexamine this question by assessing the extent of HGT among core orthologous genes using a novel statistical method based on statistical comparisons of tree topology. We apply the method to 40 microbial genomes in the Clusters of Orthologous Groups database over a curated set of 297 orthologous gene clusters, and we detect significant HGT events in 33 out of 297 clusters over a wide range of functional categories. Estimates of positions of HGT events suggest a low mean genome-specific rate of HGT (2.0%) among the orthologous genes, which is in general agreement with other quantitative of HGT. We propose that HGT events, even when relatively common, still leave the treelike history of phylogenies intact, much like cobwebs hanging from tree branches.

Introduction

The role of horizontal gene transfer (HGT) in speciation, adaptation, and evolution of life on earth has been studied intensively [1], and there has been a growing body of evidence of transfers of genes among species [2–4] and transfers from organelles to nuclei [5–7]. Whole genome analyses of different prokaryotes have been thought to indicate rampant HGTs [8,9] and suggest that HGT plays a pivotal role in prokaryotic evolution, producing dynamic and mosaic genomes. The speculation [10] that even genes involved in transcription and translation might have been subject to HGT has also led to the suggestion that HGT should be considered the essence of phylogeny and that HGT might have eroded the organismal genealogical trace. Therefore, life history cannot be properly represented by the traditional treelike form, but rather by a netlike form [4,11–13].

One of the main unresolved issues in the debate is the estimation of HGT frequency [14] and its impact on phylogeny [15]. Commonly used methods for detecting HGT are based on observations of (1) atypical gene sequence composition [16,17]; (2) unexpected rankings for sequence similarity among homologs [18]; and (3) incongruence among phylogenetic trees [e.g., 7]. Studies based on sequence characters suggested HGT frequency at 24% in Thermotoga [2] and a range up to 17% among different prokaryotes [19,20]. Conflicts between the 16S rRNA tree and other gene trees have been frequently reported [e.g., 21]. These findings have led to the ongoing debate about the impact of HGT on phylogeny. Some researchers believe that HGTs are so frequent that a core of nontransferable genes might not exist and that phylogeny in treelike form has little utility [20,22]. Other researchers, however, believe that HGTs constitute only minor interference when inferring phylogeny and propose that methods for inferring HGT have various problems leading to an overestimation of its frequency [1].

For example, a previous study shows that different methods for estimating HGT gave different sets of HGT candidates when applied to the same genome [23]. Meanwhile, it has been proposed that a phylogeny could be sufficiently retrieved via a core of genes that may be resistant to HGT [24,25]. A congruent phylogenetic structure inferred from different genes was proposed as further evidence to buttress this argument [26–29].

As pointed out in Daubin and Ochman [30], there is a difference between assessing genetic transfer among elements with some recognizable homology or orthology to sequences in other genomes and assessing genetic transfer in the entire genome, which may have indeed incorporated significant foreign genetic material, through processes such as selection for pathogenicity [19]. Thus, the key question is whether sequences with recognizable homologs in a significant number of genomes show high levels of HGT and whether HGT’s effects are sufficient enough to impede the building of branching phylogenetic history [31]. HGT events lead to incongruent phylogenies for different genetic elements. But at the same time, incongruence in phylogenies can be caused by a list of factors, such as artifacts of phylogenetic reconstruction or other biological sources [32,33].

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Germescale Horizontal Gene Transfer

**Results**

Figure 1 shows our computational flow, which we briefly describe here and in detail in the Materials and Methods section. First, we use the COG database to assemble a set of high-quality orthologous groups for tree inference. Then we build the W-G tree that represents the best treelike history of the genome. The key point in this procedure is that we explicitly test the alternative hypothesis of HGT rather than merely a rejection of congruence. In the end, for those genes that display statistically significant evidence of horizontal transfer, we estimate the position of the transfer events based on the W-G tree and compute genome-specific and gene-specific rates of transfer.

**High-Quality Gene Groups and the W-G Tree**

The COG database, built by all-versus-all sequence comparisons, covers 43 microorganisms, including complete genomes of bacteria, Archaea, and *Saccharomyces cerevisiae*, in the initial version, which we used for this study. Our stringent high-quality COG selection procedure described in the Materials and Methods section resulted in the retention of 297 COG entries out of the original 3,852, which cover 40 genomes (Table 1). On average, each high-quality COG covered 16.5 genomes, representing both universally distributed genes and lineage-specific genes. Rather than use any single set of sequences (e.g., rRNA) to approximate the W-G tree, we used the median tree estimator designed by Kim and Salisbury [34], which is a robust estimator that attempts to overcome major genetic distortions such as HGTs. The high-quality COG entries were used to construct the median tree estimate (as shown in Figure 2) with bootstrap values obtained from bootstrap resampling of the input COG entries (the branches with less than 50% bootstrap support were collapsed to improve the reliability of later analysis).

In this unrooted W-G tree, the three domains of life are monophyletic with high bootstrap values. Also, the tree strongly supports the monophyly of *Chlamydiales*, *Spirochaetes*, low G+C gram-positives, high G+C gram-positives, and *β*, *γ*, and *δ*-Proteobacteria. The artificial attraction of long branches of Archaea and hyperthermophilic bacteria does not appear, with the grouping of *Aquifex aeolicus* and *Thermotoga maritima* into the bacterial domain, which is consistent with recent studies [37]. However it should be noted that other authors suggest *A. aeolicus* should group with Proteobacteria based on shared putative indels.
protein domain architecture, and membrane structure [38–41], and the grouping remains controversial. Although most of the branches are supported by high bootstrap values, it is worth noting that this tree is partially unresolved, as branches with bootstrap values lower than 50% have been collapsed. Hence, this tree neither informs us on the basal position of the bacterial domain, nor informs us much on the basal branching patterns of archaean phylogeny, which results in some loss of power for detecting HGT events across these lineages (see also Discussion). Outside of this, two possible artifacts are the basal position of *Halobacterium* at the archaean domain, which has been suggested to be affected by a large number of HGT events from bacterial origin [42,43], and the grouping of *ε*-Proteobacteria with *Chlamydiales* and *Spirochaetes*, which are commonly seen in literature [27].

### Statistical Inference of HGT and Power Test

HGT events for a particular gene or sequence can be detected using phylogenetic methods that compare the estimated gene tree for the candidate sequences against the other gene trees or against some candidate tree that represents the history of the genomes. Mathematical tree distance metrics can be used to measure the discrepancy between two trees. However, two trees may be different because of an HGT event or other reasons, such as noise in the data, compositional bias, hidden gene duplication, gene loss, and so on. Thus, one possible approach is to ask whether the discrepancy between two trees can be more easily explained by simple branch-exchange events (which would be evidence of HGT)—i.e., to explicitly consider the HGT as an alternative hypothesis.

Suppose we have two trees, A and B. If A and B differ by branch-transfer events, they should share a common subtree, wherein the transferred branches have been removed. A bound on the size of such a shared common subtree can be computed using an algorithm called maximum agreement subtree (MAST [44]). Moreover, the difference between A and B can also be measured by the number of branch edges shared by the two trees, computed by a measurement called

| Genome | Number of COG Entries |
|--------|------------------------|
| Eco    | 235                    |
| Vch    | 165                    |
| Pae    | 155                    |
| Prmu   | 140                    |
| EcZ    | 136                    |
| Hin    | 132                    |
| Ctz    | 130                    |
| Mlo    | 130                    |
| BsU    | 127                    |
| NmA    | 126                    |
| Bhs    | 125                    |
| Nme    | 125                    |
| Xfa    | 118                    |
| Dra    | 114                    |
| Gje    | 112                    |
| Mtu    | 112                    |
| Syn    | 111                    |
| Hbs    | 101                    |
| MsA    | 99                     |
| Aae    | 99                     |

Abbreviations for names of species defined in Table 3.

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**Figure 2. The W-G Tree Based on the Median Tree Algorithm**

A subset of high-quality COG entries, which covers at least seven genomes, was used to build the W-G tree (see Materials and Methods). Branches with bootstrap scores less than 50% were collapsed into the polytomous form. Three domains of life are shown as (A) Archaea, (B–J) Bacteria, and (K) Eukaryote. Species are labeled with different colors based on their inferred HGT rates: red, >4%; yellow, 3%–4%; pink, 2%–3%; blue, 1%–2%; green, <1%. Taxonomy labels are (A) Euryarchaeae, (B) Proteobacteria, (C) Chlamydia, (D) Spirochaetes, (E) Thermotogae, (F) Aquificae, (G) Actinobacteria, (H) Deinococcus, (I) Cyanobacteria, (J) Firmicutes, and (K) Fungi.

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symmetric difference (SD) metric (also known as Robinson-Foulds metric [45]). Simply, the SD metric computes the number of different splits, regardless of whether or not the difference in the two trees can be explained by a branch switch (and thus putative HGT). Hence, the combination of MAST distance and SD distance between tree A and tree B can be interpreted in terms of putative HGT events (Figure 3). If both MAST and SD distance values are low, then the two trees are not likely to be statistically different. If both MAST and SD values are large, then they may be different, but the difference is not easily explained by an HGT event. The disparity could be due to many other factors (including, of course, HGT). On the other hand, if the two trees differ by a large SD value but are generally similar with a small MAST score, this suggests that the difference can be best explained by putative HGT events. The last case, large MAST distance but low SD distance, cannot occur due to algorithmic reasons.

Taking this into account, we developed a hypothesis test for HGT, using the difference between the normalized values of the two metrics, which we denote by $\gamma$ (see Materials and Methods). We computed the significance of an observed $\gamma$ value by generating a nonparametric null distribution based on randomly bootstrapped gene trees (see Materials and Methods). In our tree topology-based HGT test, we do not explicitly take branch length into account; however, the bootstrap distribution implicitly allows the incorporation of branch-specific confidence. HGT was inferred when the observed $\gamma$ was significant with the $p$-value below the 5% level. The power of this procedure in detecting HGT was tested with a simulation study (detailed in Materials and Methods). These simulation studies applied to each COG showed that on average we were able to detect HGT events at 53.8%, 70.0%, and 77.3%, respectively, for one, two, and three HGT events in a COG tree using the 5% significance level, 1,764 out of the 14,004 pairs were significant under our hypothesis test for HGT. We expected different $p$-values for the significance level to affect the power of the test, with larger critical $p$-values tending to more liberally infer HGT events. We investigated the effect of the significance levels on the inferred number of HGT events. The number of COG entries inferred to contain HGT events does not increase dramatically as the cutoff significance value increases (Figure 5). Thus, assuming the standard 5% significance level seemed acceptable to guard against type I error; more liberal values are not expected to significantly change our conclusions about genomic rates of HGT. At the significance level of 0.05, we inferred that 33 out of 297 COG entries (i.e., 11.1%) contain putative HGT events (Table 2). Below, we will call the COG entries with statistically significant HGT events hCOGs. These hCOGs cover a wide range of functional categories as annotated in the COG database [36]. Figure 6 shows the relative frequency of hCOGs within each functional category and aggregated into broader functional categories. We used Fisher’s exact test (two-sided) [46] to determine the relationship between the presence of HGT and functional categories. Only one functional category H (coenzyme metabolism) stood out as having a significantly higher (at 0.05 significance level) amount of HGT events. This is in agreement with HGT cases found in literature [4] and supports the speculation that so-called operational genes are more prone to HGT than so-called informational genes [24,47].

**HGT Estimation via Comparisons between Each Gene Tree and the W-G Tree**

The hypothesis test described above was applied to each of the 297 COG gene trees against the W-G tree. We expected different $p$-values for the significance level to affect the power of the test, with larger critical $p$-values tending to more liberally infer HGT events. We investigated the effect of the significance levels on the inferred number of HGT events. The number of COG entries inferred to contain HGT events does not increase dramatically as the cutoff significance value increases (Figure 5). Thus, assuming the standard 5% significance level seemed acceptable to guard against type I error; more liberal values are not expected to significantly change our conclusions about genomic rates of HGT. At the significance level of 0.05, we inferred that 33 out of 297 COG entries (i.e., 11.1%) contain putative HGT events (Table 2). Below, we will call the COG entries with statistically significant HGT events hCOGs. These hCOGs cover a wide range of functional categories as annotated in the COG database [36]. Figure 6 shows the relative frequency of hCOGs within each functional category and aggregated into broader functional categories. We used Fisher’s exact test (two-sided) [46] to determine the relationship between the presence of HGT and functional categories. Only one functional category H (coenzyme metabolism) stood out as having a significantly higher (at 0.05 significance level) amount of HGT events. This is in agreement with HGT cases found in literature [4] and supports the speculation that so-called operational genes are more prone to HGT than so-called informational genes [24,47].

**HGT Estimation via Comparisons among Gene Trees**

One problem with the above procedure is that the results are sensitive to the particular reference tree, i.e., the W-G tree. To overcome this problem, we next tested for possible HGTs by all pairwise comparisons of 297 COG entries. However, the COG entries do not all share the same taxa, and when the number of shared taxa is too low, we do not have sufficient power to estimate HGTs. Thus, we compared 14,004 pairs of gene trees that contained greater than or equal to six shared taxa. The same hypothesis test for HGT was applied to these pairs of gene trees. With the significance cutoff at 5% level, 1,764 out of the 14,004 pairs were significant under our test, suggesting that 12.6% of the tree pairs contain two trees significantly different from each other in terms of HGT. We then used this fraction to calculate the percentage of hCOGs. In pairwise comparisons, we have the following four cases: (1)
neither tree has HGT events; (2) the first tree has HGT events; (3) the second tree has HGT events; and (4) both trees have HGT events. Suppose in our collection, we have $x$ percent of COG entries with detectable HGT events. Then for a given COG, if it is a normal COG, we would expect it to test significantly different in $x$ percent of the comparisons; if it is an hCOG, it should test differently for all of the comparisons. By considering such pairwise tests we can estimate the percentage of the COG entries with detectable HGT events (see Materials and Methods for more details). In our case, we estimate that 13% of COG entries may contain HGT, which is not far from the estimate (11.1%) obtained from W-G tree comparison. The pairwise test may have greater power for discrimination since most of the gene trees are fully resolved compared to our W-G tree.

**HGT Frequency in 40 Microbial Genomes**

For each of the 33 hCOGs that were identified based on the comparison between each COG tree and the W-G tree, we estimated the positions of putative transfers by using an exhaustive searching procedure (see Materials and Methods for details). This allowed us to compute the genome-specific rate of HGT events among the high-quality COG entries as an estimate of the overall rate of HGT events per genome. Figure 2 shows a colored annotation of the genome-specific rate of HGT laid on top of the W-G phylogeny. Table 3 lists the HGT rate per each genome and the particular COG entries involved in the HGT. The distribution of HGT events along the W-G phylogeny shows no obvious pattern of concentrated events: genomes with high rates of HGT events seem evenly scattered across the phylogeny. As listed in Table 3, the frequency of HGT events ranges from 0% in *Chlamydia*. 

**Figure 4. Power of the $\gamma$ Test in Detecting HGT**

Random SPR operations were applied to each COG tree to assess the power of the $\gamma$ test. The figures show the power values plotted against the taxon numbers in the COG entries for 1, 2, and 3 SPR changes. DOI: 10.1371/journal.pbio.0030316.g004

**Figure 5. The Relationship between Detecting COG Entries with HGT and the p-Values**

Dotted curve: the number of COG entries detected to contain HGT at given p-value cutoffs. Straight line: the number of COG entries identified to contain HGT merely by chance, based on given p-value cutoffs. When the cutoff for p-value increases, the number of COG entries that might contain HGT increases, as one would expect. However, the small slope of this curve compared with the line of null hypothesis suggests that the frequency of HGT does not change dramatically, even in a relatively flexible p-value range. DOI: 10.1371/journal.pbio.0030316.g005

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pneumoniae and six other genomes to 6.7% in Methanobacterium thermoautotrophicum. The rates of HGT in Aeropyrum pernix, Xylella fastidiosa, and some other archaeal organisms, which are notable for their dynamic genome evolution, are relatively high in our result; while the rates for some intensely studied organisms, such as Escherichia coli, are not as high as previously reported [17,20]. Of the top five genomes in our list, all except the M. thermoautotrophicum rank highly for rates of HGT in other surveys [e.g., 17,48,49]. M. thermoautotrophicum, which seems to be typically at the middle of HGT rates in other surveys, stands out in our assay. One possibility is that Dufraigne et al. [48] found M. thermoautotrophicum to have unusually long stretches of putative HGT tracks—and perhaps offering more power by our topology-based test. The mean rate of HGT, 2.0%, among core genes per genome, is considerably lower than those reported in other studies, but the result is consistent with some phylogenetic studies focusing on smaller sets of species [50].

### Table 2. List of Transferred Genes

| Functional Category | COG Accession Number | Number of Genomes in the COG Entry | Protein Name |
|---------------------|----------------------|-----------------------------------|--------------|
| J                   | COG1549              | 8                                 | Queuine tRNA-ribosyltransferases, contain PUA domain |
| J                   | COG1746              | 9                                 | tRNA nucleotidyltransferase (CCA-adding enzyme) |
| L                   | COG1059              | 6                                 | Thermostable 8-oxoguanine DNA glycosylase |
| L                   | COG1423              | 7                                 | ATP-dependent DNA ligase, homolog of eukaryotic ligase III |
| M                   | COG0677              | 10                                | UDP-N-acetyl-D-mannosaminurionate dehydrogenase |
| M                   | COG2943              | 6                                 | Membrane glycosyltransferase |
| N                   | COG1955              | 8                                 | Archaeal flagella assembly protein J |
| O                   | COG2039              | 7                                 | Pyrrolidone-carboxylate peptidase (N-terminal pyroglutamyl peptidease) |
| P                   | COG1613              | 9                                 | ABC-type sulfate transport system, periplasmic component |
| C                   | COG1062              | 11                                | Zn-dependent alcohol dehydrogenases, class III |
| C                   | COG1282              | 14                                | NAD/NADP transhydrogenase β subunit |
| C                   | COG1894              | 14                                | NADH-ubiquinone oxidoreductase, NADH-binding (51 kDa) subunit |
| E                   | COG0411              | 6                                 | ABC-type branched-chain amino acid transport systems, ATPase component |
| E                   | COG0646              | 12                                | Methionine synthase I (cobalamin-dependent), methyltransferase domain |
| E                   | COG1166              | 13                                | Arginine decarboxylase (spermidine biosynthesis) |
| E                   | COG2957              | 8                                 | Peptidylarginine deiminase and related enzymes |
| F                   | COG1972              | 7                                 | Nucleotide permease |
| G                   | COG1023              | 8                                 | Predicted 6-phosphogluconate dehydrogenase |
| G                   | COG3265              | 9                                 | Gluconate kinase |
| H                   | COG0029              | 18                                | Aspartate oxidase |
| H                   | COG0379              | 24                                | Quinolinate synthase |
| H                   | COG1010              | 9                                 | Preconin-38 methylase |
| H                   | COG1635              | 9                                 | Flavoprotein involved in thiazole biosynthesis |
| H                   | COG1995              | 15                                | Pyridoxal phosphate biosynthesis protein |
| H                   | COG2227              | 12                                | 2-polypropyl-3-methyl-5-hydroxy-6-metoxy-1,4-benzoquinol methylase |
| H                   | COG2875              | 10                                | Preconin-4 methylase |
| R                   | COG1242              | 10                                | γ-Glutamylcysteine synthetase |
| R                   | COG2130              | 7                                 | Predicted Fe-S oxidoreductase |
| S                   | COG1288              | 8                                 | Predicted membrane protein |
| S                   | COG1584              | 8                                 | Predicted membrane protein |
| S                   | COG1636              | 13                                | Uncharacterized protein conserved in bacteria |
| S                   | COG2326              | 6                                 | Uncharacterized conserved protein |

CCG entries that are involved with HGT were identified based on our test, with p ≤ 0.05. C, energy production and conversion; E, amino acid transport and metabolism; F, nucleotide transport and metabolism; G, carbohydrate transport and metabolism; H, coenzyme metabolism; J, translation, ribosomal structure, and biogenesis; L, DNA replication, recombination, and repair; M, cell envelope biogenesis, outer membrane; N, cell motility and secretion; O, posttranslational modification, protein turnover, chaperones; P, inorganic ion transport and metabolism; R, general function prediction only; S, function unknown.

*From the COG database.*

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### Discussion

Our main results show that HGT events can be inferred in only 33 out of the 297 COG entries studied (11.1%) in a comparison against a reference tree and 13% in pairwise comparisons among the tree pairs. The estimated rates of HGT in different genomes are between 0% and 6.7%, with an average of 2.0% among the 40 genomes studied here. There are several factors to consider in this rate estimation. First, as noted in Daubin et al. [51], one of the key questions is the rate of HGT events within those genes that can be orthologously compared to one another reliably (even if they are part of a paralogous family). The use of the COG database and our procedure for retaining only high-quality COG entries mean that our rate computation is limited to such gene sets. Thus, similar to Lerat et al. [28], where very few conflicts among gene trees of widespread single-copy orthologs in γ-Proteobacteria were found, our computed rate of HGT is only for
those genes for which reliable orthologous copies can be found in multiple genomes. This might underestimate the HGT rates by ignoring sporadically distributed genes shared by only two or three genomes and those orthologous groups that cannot be reliably assembled via the mutual best-hit approach. On the other hand, for genes from large paralogous families or those only found in a few genomes, reliable assessment is impossible for either HGT or vertical transmission.

Second, we used a specific statistical test where, rather than simply asking whether two gene trees are significantly different from each other, we asked whether the trees are different and can be significantly better explained by horizontal transfer. With this in mind, we conducted a test for a specific alternative hypothesis of HGT rather than the broad rejection of simple tree congruence. A specific test of alternative hypothesis provides additional protection against false rejection of the null hypothesis. Our simulation studies suggest that our test retains reasonable power for detecting HGT events despite this additional precaution. Recently, Novichkov et al. [35] carried out a test for abnormal pairwise divergence patterns similar to our test (but with a stronger assumption of a molecular clock) and found possible HGT in approximately 17%–30% of the COG entries. The fact that we specifically test for positive evidence of HGT and allow more relaxed non-clock-like evolution may explain this discrepancy.

Third, the significance level of the hypothesis test can change the rate estimates. However, within the range of values examined, the estimated numbers of HGT events do not significantly change with increased risk of false rejection. For example, if we increase the significance value to 0.1, then we obtain 39 out of 297 COG entries (13.1%) that may contain HGT events, which is still within the lower range of values reported by others.

Fourth, our statistical test has greater power for phylogenetically distant transfer events compared to proximal transfers. This is because the tree comparison metric SD and MAST differ the most when a tree involves a branch

### Table 3. Frequency of HGT in 40 Genomes and List of Transferred Genes

| Organism                        | HGT Frequency | COG Entries with HGT |
|---------------------------------|---------------|-----------------------|
| Methanobacterium. thermoaerotrophicum | 6.74% COG0677, 1549, 1746, 1010, 1635, 1584, 0379 |                     |
| Synchocystis                    | 5.56% COG0209, 1282, 1894, 1062, 0379, 1010, 1995 |                     |
| Xylella. fastidiosa             | 5.51% COG0029, 1613, 1636, 2227, 1995, 0379, 1166 |                     |
| Aquifex. aeolicus               | 5.39% COG0209, 1059, 1636, 1023 |                     |
| Aeropyrum. pernix               | 4.73% COG1746, 1955, 1635, 1423 |                     |
| Pasteurella. multocida          | 3.57% COG0209, 2875, 3263, 0379, 1010 |                     |
| Helicobacter. pylori            | 3.51% COG0209, 2875, 3263, 0379, 1010 |                     |
| Staphylococcus. aureus          | 3.37% COG0209, 2875, 3263, 0379, 1010 |                     |
| Vibriob. cholerae               | 2.33% COG0209, 2875, 3263, 0379, 1010 |                     |
| Neisseria. meningitidis         | 1.82% COG0209, 2875, 3263, 0379, 1010 |                     |
| Neisseria. meningitidis         | 1.39% COG0209, 2875, 3263, 0379, 1010 |                     |
| Bacillus. halodurans            | 1.32% COG0209, 1635, 2130 |                     |
| Halobacterium. sp.              | 1.25% COG0209, 1635, 2130 |                     |
| Neisseria. meningitidis         | 1.19% COG0209, 1635, 2130 |                     |
| Neisseria. meningitidis         | 1.19% COG0209, 1635, 2130 |                     |
| Neisseria. meningitidis         | 1.19% COG0209, 1635, 2130 |                     |
| Neisseria. meningitidis         | 1.19% COG0209, 1635, 2130 |                     |

HGT frequency was calculated as the percentage of the number of HGT genes in one genome out of the total number of genes of the genome from 297 COG entries that we surveyed. COG accession numbers are from the COG database (http://www.ncbi.nlm.nih.gov/COG/). Transferred branches in each gene tree are identified based on tree comparison (see Materials and Methods).

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transfers among distant taxa. For nearest-neighbor branch transfers, both methods yield the same value and thus cannot distinguish simple statistical error versus potential HGT event. Hence, if the HGT events frequently involve sister taxa, our estimate of HGT rates will be an underestimate. It is not clear whether HGTs should be more common between close lineages [52]. The mechanism and potential effect of HGT events are different from recombination and hybridization, and therefore it is difficult to assert a lineage distance effect. For example, HGT between distantly related taxa might be argued to be more likely purely due to the increased elapsed time.

Finally, we excluded the high SD and high MAST as cases where HGT events cannot be decided with high confidence. We tested the significance of both high SD and MAST scores using the bootstrap procedures described above. We found just 44 out of 297 cogs (14.8%) that have significantly high SD and MAST values but do not have significantly low $\gamma$ for both the W-G tree comparison and the pairwise comparison. We are wary of treating such cases as HGT events, but regardless, these cases can be considered to add to an upper bound to HGT estimation. But we believe that such HGT events will be very difficult to detect based on gene genealogies alone. A reliable test would require more densely sampled taxa or other supporting evidence such as sequence compositional characteristics.

We have previously shown by simulation methods that even when there are large-scale HGT events (several events per gene), there remains a recognizable tree that represents the consistent tree-like evolution of the majority of the genes and lineages [34]. One way to consider this is to imagine a very large tree, say 10,000 taxa, and some large number of potential “units” of HGT, say 10,000 such elements per genome. Even if each such element had, say, 1,000 actual HGTs across the 10,000 taxa, if we overlay the 10,000 trees on top of one another, all the HGTs will appear as extremely thin connections like cobwebs, and we will see a strong image of a backbone tree. More precisely, consider the relative distance between two taxa as estimated by a set of $n$ genes. Assume that our estimators are perfect; we can obtain exact scaled distance estimates such that we can estimate the absolute time of separation of the two cell lineages. Let the true time of separation be $T^0$ and assume that an HGT event along the two lineages yields some variant time estimate, larger if the HGT event brings in a homologous copy from outside the extent of the two lineages, or smaller if the HGT event involves homologous copies from inside the two lineages. Say, for the $n$ genes, $k$ of them experienced horizontal transfer; then we have $(n-k)$ values of $T^0$, and we need at least as many coincident draws for the HGT time estimates to set some other time estimate to be the modal value—an extremely unlikely event given the possible variant time points in a diverse tree. Thus, while an HGT event can considerably distort the treelike structure of genomic information, there still remains a distinct tree representing the modal information lineage.

Our W-G tree described here is an explicit estimation of this “modal lineage” tree. The estimation of such a modal lineage tree allows us to use explicit tree-based techniques to estimate deviations, i.e., HGT events. When we dissect the signals based on phylogenetic methods with an explicit hypothesis test for HGT, we find that HGT is not as widespread as previously believed [53,54]. Furthermore, the estimated degree of HGT is consistent no matter whether we base it on the modal lineage tree or on pairwise comparisons. The list of HGT candidates is far from being long enough to be called “rampant” for orthologous gene sets, and the overall rates are similar to those found in other studies using phylogenetic methods [28]. We are far from claiming that the reconstruction of the history of life is trivial; however, new developments in orthologous clustering, multiple sequence alignment, tree construction algorithms, and tree-rooting problems may shed more light on the impact of HGT on phylogeny and help us understand the multiple forces of prokaryotic evolution.

Materials and Methods

Input data preparation—Selecting high-quality COG entries. We obtained a set of putative orthologous gene clusters from the COG database (the initial version [30]; ftp://ftp.ncbi.nih.gov/pub/COG/old/). These data were processed in the following way to increase the reliability of later analysis. (We also excluded three small genomes from the original COG database, as the number of high-quality COG entries covering these genomes was too small.) (1) Best-hit confirmation: all-against-all BLAST searching was redone for all 43 genomes, and every “two-way or one-way best-hit” status for each pair of proteins was tested. Protein members that were not the top hits for any other proteins were removed from the dataset. (2) Removal of large protein families: some of the COG entries are superprotein families, which have gone through extensive gene duplication. They are not easy to use in building reliable sequence alignments and are not suitable for supertree construction. We excluded those large COG entries where the number of sequences exceeds the number of genomes by more than 2.5-fold. (3) Estimation of COG quality by checking BLAST sequence alignments; the quality of each COG was assessed based on the pairwise BLAST $e$-values and lengths of the significant aligned regions. We obtained 511 COG entries from which all the $e$-values of pairwise BLAST scores were lower than $10^{-50}$ and whose proportion of high-scoring aligned regions to the whole protein sequences was greater than 50%. (4) Building distance matrices: we first generated multiple sequence alignments using CLUSTALW [53], and then used PHYLIP [56] to calculate distance matrices based on the alignments. PHYLIP’s command tool prodist was used with default setting of Dayhoff PAM matrix was used to make our calculations. Gap regions in the alignments were dropped because they might not have been aligned properly. (5) The list of HGT candidates is far from being long enough to be called “rampant” for orthologous gene sets, and the overall rates are similar to those found in other studies using phylogenetic methods [28]. We are far from claiming that the reconstruction of the history of life is trivial; however, new developments in orthologous clustering, multiple sequence alignment, tree construction algorithms, and tree-rooting problems may shed more light on the impact of HGT on phylogeny and help us understand the multiple forces of prokaryotic evolution.

Building gene trees and the W-G tree. For each of the 297 COG entries we constructed a gene tree by computing a neighbor-joining tree (PAUP* [57], using the distance matrix computed as described above. For each gene tree, 1,000 bootstrap replicates were computed by bootstrap sampling from the original sequences and computing a replicate distance matrix. We computed a consensus tree for the bootstrap replicates according to the majority rule; this was used as the gene tree estimate. We then applied the median tree algorithm [34] to 230 out of the 297 COG entries to build the W-G tree estimate. First, to describe in brief, given a set of distance matrices, the algorithm computes the median of normalized distances as a robust estimate of the true evolutionary distance. It has been shown to be particularly useful for estimating the genome tree when individual genes undergo HGT events. The detailed procedure follows: (1) Data selection: although there were 297 high-quality COG entries, those that covered only a small number of genomes could render the normalization process unstable. Therefore, we used a subset of 230 high-quality COG entries that covered at least seven genomes for the W-G tree estimate. (2) Normalization of distance matrices of COG entries: the median tree
null distribution described next is also based on the normalized statistics, thus controlling for taxon sampling effects of tree topologies. The null distribution of $\gamma$ was obtained by a randomization procedure. For each COG, 2,000 bootstrap trees were generated from the original sequence alignment. We divided them into 1,000 pairs of trees, for each of which the statistic $\gamma$ was calculated. The distribution of 1,000 $\gamma$-values computed in this manner represents the null distribution in which the tree differences are not due to HGT. For each COG, $\gamma$ was calculated for the gene tree against the W-G tree described above infers presence and absence of HGT events for a given COG. For each COG that tested positive for HGT events, we identified the particular branches of transfer by exhaustive enumeration of possible subtree matches. Since the MAST score gives the number of taxa needed to make the two trees identical, we exhaustively searched for all combinations of branch prunings to find the “troublesome” branches. When there is only one way of pruning branches to make the two trees congruent to each other, those pruned branches are identified as HGT events. However, on a limited number of occasions, there was more than one way of pruning the branches. We treated those branches as equally probable transfers and assigned them a probability weight based on the number of possible prunings. For each genome, the total number of putative HGT events was summed, and the rate of HGT was calculated based on the number of HGT events that contained genes from that genome.

**Supporting Information**

**Accession Number**

The COG database (http://www.ncbi.nlm.nih.gov/COG/) accession number for the reference COG is COG0541.

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Aquifex pyrophilus: Genomewide phylogenetic analyses identified a reliable set of Implications for the evolution of the Aquificales paradox. Genome Biol 5: R17.

Halobacterium

Novichkov PS, Omelchenko MV, Gelfand MS, Mironov AA, Wolf YI, et al. (2001) A tree obscured by vines: Horizontal gene transfer among bacterial genomes. Mol Biol Evol 18: 1884–1894.

Kim J, Salisbury BA (2001) A tree obscured by vines: Horizontal gene transfer among bacterial genomes. Mol Biol Evol 18: 1884–1894.

Slowinski JB, Page RD. (1999) How should species phylogenies be inferred from sequence data? Syst Biol 48: 814–825.

Kyrpides NC, Olsen GJ (1999) Archaeal and bacterial hyperthermophiles: Horizontal gene exchange or common ancestry? Trends Genet 15: 298–299.

Maddison WP (1997) Gene trees in species trees. Syst Biol 46: 523–526.

Slonkins JB, Page RD. (1999) How should species phylogenies be inferred from sequence data? Syst Biol 48: 814–825.

Kim J, Salisbury BA (2001) A tree obscured by vines: Horizontal gene transfer and the median tree method of estimating species phylogeny. Pac Symp Bioinform 6: 571–582.

Novichkov PS, Omelchenko MV, Gelfand MS, Mironov AA, Wolf YI, et al. (2004) Genome-wide molecular clock and horizontal gene transfer in bacterial evolution. J Bacteriol 186: 6575–6585.

Tatusov RL, Natale DA, Garkavtsev IV, Tatusova TA, Shankavaram UT, et al. (2001) The COG database: New developments in phylogenetic classification of proteins from complete genomes. Nucleic Acids Res 29: 25–29.

Brochier C, Philippe H (2002) Phylogeny: A non-thermophilius ancestor for bacteria. Nature 417: 244.

Gupta RS, Griffiths E (2002) Critical issues in bacterial phylogeny. Theor Biol 211: 423–434.

Griffiths E, Gupta RS (2004) Signature sequences in diverse proteins provide evidence for the late divergence of the order Aquificales. Int J Syst Evol Microbiol 52: 7–16.

Iyer LM, Koonin EV, Aravind L (2004) Evolution of bacterial RNA polymerase: Implications for large-scale bacterial phylogeny, domain accretion, and horizontal gene transfer. Gene 335: 73–88.

Cavalier-Smith T (2002) The neomuran origin of archaeabacteria, the negibacterial root of the universal tree and bacterial megaclassification. Int J Syst Evol Microbiol 52: 7–16.

Brochier C, Forterre P, Gribaldo S (2004) Archaeal phylogeny based on proteins of the transcription and translation machineries: Tackling the Methanopyrus kandleri paradox. Genome Biol 5: R17.

Kennedy SP, Ng WV, Salzberg SL, Hood L, DasSarma S (2001) Understanding the adaptation of Halobacterium species NRC-1 to its extreme environment through computational analysis of its genome sequence. Genome Res 11: 1641–1650.

Goddard W, Kubicka E, Kubicki G, McMorris FR (1994) The agreement metric for labeled binary trees. Math Biosci 103: 215–226.

Robinson DF, Foulds LR (1981) Comparison of phylogenetic trees. Math Biosci 53: 131–147.

Conover WJ (1999) Practical nonparametric statistics, 3rd ed. New York: Wiley. 584 p.

Harris JK, Kelley ST, Spiegelman GB, Pace NR (2003) The genetic core of the universal ancestor. Genome Res 13: 407–412.

Dufraine C, Fertil B, Lespinats S, Girou A, Deschavanne P (2005) Detection and characterization of horizontal transfers in prokaryotes using genomic signatures. Nucleic Acids Res 33(1): e6. Available: http://pubmedcentral.ncbi.nlm.nih.gov/articlerender.fcgi?tool=pubmed&pubmedid=15653627. Accessed 19 July 2005.

Michalato-Soto A, Moreno-Hagelsieb G, Vinuesa P, Christea JA, Collado-Vides J (2004) Successful lateral transfer requires codon usage compatibility between foreign genes and recipient genomes. Mol Biol Evol 21: 1884–1894.

Ochman H, Lawrence JG (1998) Molecular archaeology of the bacterial rpoBC operon and extremely thermophilic bacteria. J Mol Evol 48: 529–541.

Bapteste E, Boucher Y, Leigh J, Doolittle WF (2004) Phylogenetic reconstruction and lateral gene transfer. Trends Microbiol 12: 406–411.

Ragan MA (2001) On surrogate methods for detecting lateral gene transfer. FEMS Microbiol Lett 201: 187–191.

Jain R, Rivera MC, Lake JA (1999) Horizontal gene transfer among genomes: The complexity hypothesis. Proc Natl Acad Sci U S A 96: 3801–3806.

Eisen JA (2000) Horizontal gene transfer among microbial genomes: New insights from complete genome analysis. Curr Opin Genet Dev 10: 606–611.

Marte-Tailliez O, Brochier C, Forterre P, Philippe H (2002) Archaeal phylogeny based on ribosomal proteins. Mol Biol Evol 19: 631–639.

Daubin V, Gouy M, Perriere G (2002) A phylogenomic approach to bacterial phylogeny: Evidence of a core of genes sharing a common history. Genome Res 12: 1080–1090.

Lartet E, Daubin V, Moran NA (2003) >From gene trees to organismal phylogeny in prokaryotes: The case of the y-Proteobacteria. PLoS Biol 1: e19. DOI: 10.1371/journal.pbio.0010019.

Durbin RB, Hugenholtz MA, Bruno WJ, Snel B (2004) The consistent phylogenetic signal in genome trees revealed by reducing the impact of noise. J Mol Evol 58: 527–539.

Daubin V, Ochman H (2004) Quartet mapping and the extent of lateral transfer in bacterial genomes. Mol Biol Evol 21: 86–99.

Kyrpides NC, Olsen GJ (1999) Archaeal and bacterial hyperthermophiles: Horizontal gene exchange or common ancestry? Trends Genet 15: 298–299.

Maddison WP (1997) Gene trees in species trees. Syst Biol 46: 523–526.

Slonkis JB, Page RD. (1999) How should species phylogenies be inferred from sequence data? Syst Biol 48: 814–825.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680.

Felsenstein J (1993) PHYLIP (Phylogeny Inference Package), version 3.5c [computer program]. Seattle: University of Washington Department of Genetics. Available: http://evolution.genetics.washington.edu/phylip.html. Accessed 19 July 2005.

Swofford DL (2002) PAUP*: Phylogenetic analysis using parsimony (*and other methods), version 4 [computer program]. Sunderland (Massachusetts): Sinauer.

Semple C, Steel M (2003)Phylogenetics. New York: Oxford University Press. 259 p.