Population genomics and local adaptation in wild isolates of a microbial eukaryote

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Elucidating the connection between genotype, phenotype, and adaptation in wild populations is fundamental to the study of evolutionary biology, yet it remains an elusive goal, particularly for microscopic taxa, which comprise the majority of life. Even for microbes that can be reliably found in the wild, defining the boundaries of their populations and discovering ecologically relevant phenotypes has proved extremely difficult. Here, we have circumvented these issues in the microbial eukaryote Neurospora crassa by using a “reverse-ecology” population genomic approach that is free of a priori assumptions about candidate adaptive alleles. We performed Illumina whole-transcriptome sequencing of 48 individuals to identify single nucleotide polymorphisms. From these data, we discovered two cryptic and recently diverged populations, one in the tropical Caribbean basin and the other endemic to subtropical Louisiana. We conducted high-resolution scans for ecological genomics by resequencing mRNA from the reference strain (11). Using Bayesian phylogenies have been published that together provide broad taxon sampling across the genus (12–14), and there is a nearly complete gene deletion collection for Neurospora crassa (15). Although Neurospora is a microbe, in terms of evolution, it is very similar to more developmentally complex animals. The genus is broadly distributed but also shows patterns of geographic endemism, and both intrinsic and extrinsic barriers to reproduction are acting to maintain species boundaries (12, 16). Additionally, Dettman et al. (17) have shown that reproductive isolation arises between strains of Neurospora evolved in the laboratory under different selective regimes, suggesting that local adaptation may be an important contributor to divergence between Neurospora populations in nature. Finally, unlike yeasts, there is evidence that most species of Neurospora (including N. crassa) are highly outbred (18).

Here, we have discovered two previously unknown and recently diverged populations of N. crassa (Ascomycota) by resequencing transcriptions from 48 individuals collected from the Caribbean basin. These two populations are exposed to different local environments (subtropical vs. tropical) and exhibit “islands” of divergence in genomic regions containing genes whose functions, patterns of nucleotide polymorphism, and null phenotypes are consistent with local adaptation.

Results and Discussion

Population Genomics. We genotyped 48 isolates of Neurospora crassa from the Caribbean basin, South America, and Africa (Table SI) by identifying ≈135,000 SNPs from Illumina mRNA sequence tags. We estimated a SNP false-positive rate of 1/180,000 by sequencing mRNA from the reference strain (11). Using Bayesian clustering of allele frequencies (19) (Fig. S1) and phylogenetic inference using Bayesian methods (20) (Fig. 1), we found strong support for two cryptic populations in the dataset: one endemic to Louisiana and the other including isolates from Florida, Haiti, and the Yucatan (referred to as the Caribbean population). This genetic structure is also supported by our relatively high FST estimate of 0.19. These populations were not found by previous phylogenetic studies (12) and, in laboratory crosses, between-


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Data deposition: Sequence Read Archive SRA026962 and http://pmb.berkeley.edu/~taylorftp/Ellison_2011_SNData.txt.

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population reproductive compatibility is indistinguishable from that within populations (16).

We used a diffusion-based approach implemented in the software package \( \hat{d} \delta \hat{h} \) (21) to infer demographic parameters for these two populations under an isolation with asymmetric migration model (Fig. 2 and Table 1). To assess the goodness of fit of this model and to obtain uncertainty estimates for the demographic parameters, we used the \( \text{Neurospora} \) parameters to simulate 100 datasets in \( ms \) (22). The optimized log-likelihood and the sum-of-squares of the residuals for the real data fall within the boundaries of those values from the simulated data, implying that our model is not grossly inappropriate for our data, nor is it an example of extreme overfitting (Fig. 2B). However, as shown in the heat-maps (Fig. 2A), there is an excess of high-frequency derived alleles in our data compared with what is predicted by the model. To determine whether this pattern was an artifact resulting from the misidentification of ancestral alleles, we fit our model to two additional datasets: in the first we included two outgroups (\( \text{Neurospora tetrasperma} \) and \( \text{Neurospora discreta} \) (23, 24) and restricted our dataset to include only those SNPs for which both outgroups shared the same allele. In the second, we applied the misidentification correction that is part of the \( \hat{d} \hat{h} \) package. We still observed an excess of high-frequency derived alleles in both of these cases, suggesting that this pattern is not an artifact (Fig. S2). A similar pattern has been found in wild populations of \( \text{Arabidopsis} \) and \( \text{Oryza} \) (25, 26), and Caicedo et al. (26) found that this pattern could be explained by either a complex demographic history including population bottlenecks and high migration rates or pervasive genome-wide positive selection in the form of selective sweeps, both of which are plausible scenarios for our \( \text{Neurospora} \) populations.

We infer a relatively high population migration rate from Louisiana into the Caribbean (0.77 effective migrants per generation) and approximately one sixth that rate in the other direction (Table 1). We also inferred a relatively recent divergence time (0.4 Mya) between the two populations, in agreement with the small proportion of fixed differences (9.4%; Table 2). Although we cannot eliminate the possibility that these populations diverged in complete allopatry, this scenario seems unlikely given the high dispersal potential of fungi (27) and the fact that the Louisiana population is closer to the Caribbean population (\( \approx 1,000 \) km) than some of the Caribbean localities are to each other (\( \approx 1,000–1,600 \) km). However, the strong support for population structure from multiple methods and the migration estimates from \( \hat{d} \) both suggest that current migration is not sufficient to overcome genetic drift.

Fig. 2. Comparison of the \( \text{N. crassa} \) joint allele frequency spectrum with that expected under an isolation with migration demographic model. To determine whether our model of isolation with asymmetric migration is appropriate for our data, we used two metrics to compare the joint allele frequency spectrum for the Louisiana and Caribbean populations of \( \text{Neurospora} \) with that expected under our model: (A) visual examination of the allele frequency spectra and calculation of the residuals between model and data, and (B) log-likelihood and Pearson’s \( \chi^2 \) goodness-of-fit tests. (A) Heat-maps showing the joint allele frequency spectrum for the Louisiana and Caribbean populations compared with that expected under a simple isolation with migration model. The color of a cell at position \( [(X,Y)] \) in the matrix corresponds to the number of derived alleles (relative to \( N. \) tetrasperma; Materials and Methods) that are at frequency \( X \) in the Caribbean population and frequency \( Y \) in the Louisiana population. The residuals represent the difference in the number of alleles predicted by the model compared with that found in the data for each bin in the spectrum (red, model predicts too many; blue, model predicts too few). The cluster of blue bins in the upper right corner of the heat-map of residuals implies that the model predicts too few high-frequency derived alleles, or in other words, there is an excess of high-frequency derived alleles in the data. (B) Goodness-of-fit tests using the likelihood and Pearson’s \( \chi^2 \) statistics and based on the results of 100 coalescent simulations in \( ms \) (22) under the demographic parameters inferred for the two \( \text{Neurospora} \) populations. Better fits have likelihood and \( \chi^2 \) values closer to zero. The values from fitting the real data are shown by the red line.

Fig. 1. Unrooted Bayesian phylogeny showing cryptic population structure. The tree was inferred from 135,035 polymorphic sites. The support values are clade posterior probabilities (converted to decimal) and values less than 0.75 are not shown. Each taxon is colored according to collection locality. The pink semicircle and dashed ellipse correspond to the Louisiana and Caribbean (Florida, Haiti, Yucatan) populations, respectively. The remaining clades likely represent additional populations that we are unable to resolve given their small sample size.
Table 1. Population demographic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Point estimate</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancestral $N_e$</td>
<td>344,049</td>
<td>343,700</td>
<td>9,822.7</td>
</tr>
<tr>
<td>LA $N_e$ after split</td>
<td>1,151,961</td>
<td>1,211,000</td>
<td>488,347.4</td>
</tr>
<tr>
<td>CARIB $N_e$ after split</td>
<td>364,841</td>
<td>387,000</td>
<td>209,200.4</td>
</tr>
<tr>
<td>Divergence time</td>
<td>494,547</td>
<td>514,500</td>
<td>165,472.5</td>
</tr>
<tr>
<td>Effective migration rate: CARIB into LA</td>
<td>0.1297</td>
<td>0.1639</td>
<td>0.1376</td>
</tr>
<tr>
<td>Effective migration rate: LA into CARIB</td>
<td>0.7651</td>
<td>0.9032</td>
<td>0.4705</td>
</tr>
</tbody>
</table>

Table shows the maximum-likelihood parameter estimates for an isolation with asymmetrical migration model fitted to the joint allele frequency spectrum for the Caribbean (CARIB) and Louisiana (LA) $N$. crassa populations. Uncertainty estimates were obtained by calculating the mean of the point estimate and the corresponding SD for each model parameter from 100 datasets simulated in ms (22) using the Neurospora maximum-likelihood parameters. $N_e$, effective population size.

Comparisons with Saccharomyces. This dataset also provides an important benchmark for comparison with the recent Saccharomyces population genomic study (28) (Table 3). Our population genetic summary statistics indicate that, as predicted previously, Neurospora is much more outbred than Saccharomyces (18). Linkage disequilibrium decays to half its maximum value at a physical distance of $\approx0.78$ kb, compared with $\approx3$ kb in Saccharomyces cerevisiae and $\approx9$ kb in Saccharomyces paradoxus. Additionally, nucleotide diversity within each Neurospora population is more than twofold greater than that found in the UK population of S. paradoxus and the Wine/European cluster of S. cerevisiae. We have also used the same approach as in Liti et al. (28) to estimate the number of deleterious non-synonymous polymorphisms segregating in the Neurospora populations. Consistent with Neurospora being more outbred, we estimate that $\approx34\%$ of non-synonymous polymorphisms are deleterious in the two Neurospora populations, which is approximately half the amount estimated in Saccharomyces (28).

Finally, $\approx50\%$ of the SNPs we identified are still segregating within each of the two Neurospora populations, whereas the majority of polymorphisms identified by Liti et al. are fixed within each nonmosaic S. cerevisiae lineage. Given that their effective population sizes are approximately an order of magnitude smaller than the estimates for S. cerevisiae (29) and S. paradoxus (30), the amount of ancestral variation that remains within these two Neurospora populations implies that they are much more recently diverged than any found in the Saccharomyces study. Such recent divergence makes these populations an ideal system for the study of incipient speciation and adaptation.

Genomic Islands of Divergence Between Populations of Neurospora. To identify candidate genomic islands of divergence, we conducted sliding window estimates of three different population genetic parameters: $F_{ST}$ (31), Tajima’s $D$ (32), and Dxy (33). For each parameter, we identified empirical outliers in the 0.5% quantile. $F_{ST}$ measures relative divergence and is the most commonly used metric in studies of heterogeneous genomic divergence (34). We additionally use Dxy, a measure of absolute divergence, following the recommendation of Noor and Bennett (35). To our knowledge, Tajima’s $D$ has not been previously used for this purpose but, in principle, scans of the combined populations should produce large positive values for regions showing low within-population polymorphism and high between-population divergence, making it similar to a relative divergence measure.

We were surprised to discover little overlap between the significant regions identified by the three different metrics. Out of a total of 37 regions, only two were identified by all three metrics (Fig. S3). Interestingly, removing from analysis the sites that fell within these regions still resulted in a phylogeny with strong support for the two populations (Fig. S4). As such, these two major loci are not the sole drivers for the population structure that we observe. This finding is consistent with the results of Bayesian clustering of allele frequencies (Fig. S1) whereby the two populations were delineated only according to differences in allele frequencies and lends credence to our model in which, despite the presence of gene flow between populations, genetic drift and/or natural selection has resulted in genome-wide differences in allele frequencies between populations.

Apart from these two candidate islands of divergence, we observed little overlap between the top-scoring regions across the three different metrics of population divergence applied to the data. A total of 35 regions were called significant in some but not all analyses. To investigate these discrepancies, we examined these regions in more detail.

Genomic loci that did not achieve consensus across our tests for divergence fit into three major classes: A, block-like haplotypes that do not perfectly sort by population; B, regions where relative divergence is high but absolute divergence does not stand out from the genomic background; and C, regions where absolute divergence is high but relative divergence does not stand out from the genomic background (Fig. S5). Patterns A and B were predicted by Noor and Bennett (35) and may result from an inversion or other barrier to recombination that was segregating in the ancestral population. In pattern A, both haplotypes are still segregating in each population. In pattern B, relative divergence is high because alternate haplotypes have become fixed in each population, but absolute divergence does not stand out from the genomic background because most of the polymorphisms are still segregating in both populations. We conclude that analyses of interpolation divergence using only a single measure (e.g., $F_{ST}$) are susceptible to the identification of false positives. This result is troubling because it suggests that any such study, along with those using datasets of much lower resolution, will be unable to distinguish these misleading divergence outliers from true islands of divergence, potentially drastically overestimating the prevalence of this phenomenon (35).

Table 2. Population genetic summary statistics

<table>
<thead>
<tr>
<th>Populations</th>
<th>$F_{ST}$</th>
<th>Dxy</th>
<th>LD decay (kb)</th>
<th>D.S. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caribbean</td>
<td>0.0023</td>
<td>0.85</td>
<td>34.0</td>
<td></td>
</tr>
<tr>
<td>Louisiana</td>
<td>0.0024</td>
<td>0.70</td>
<td>33.1</td>
<td></td>
</tr>
<tr>
<td>Interpopulation</td>
<td>0.191</td>
<td>0.0029</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>S. paradoxus (U.K.)</td>
<td>0.0010</td>
<td>0.0010</td>
<td>61.0</td>
<td></td>
</tr>
<tr>
<td>S. cerevisiae (W/E)</td>
<td>0.0011</td>
<td>0.0011</td>
<td>61.0</td>
<td></td>
</tr>
</tbody>
</table>

Summary statistics for the Caribbean and Louisiana populations of $N$. crassa compared with those for the Wine/European population of S. cerevisiae (W/E) and the UK population of S. paradoxus calculated by Liti et al. (28). Dxy is the average number of pairwise differences between individuals; LD decay is the average physical distance over which the coefficient of linkage disequilibrium ($r$) decays to half its maximum value; and deleterious SNPs (D.S.) are an estimate of the number of amino acid changing polymorphisms that are deleterious, calculated as in Liti et al. (28). NA indicates that either the statistic is not applicable or the value was not calculated by Liti et al. (28).

Table 3. Summary of SNPs

<table>
<thead>
<tr>
<th>Category</th>
<th>Percentage of total SNPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixed in both populations</td>
<td>9.4</td>
</tr>
<tr>
<td>Polymorphic in both populations</td>
<td>48.7</td>
</tr>
<tr>
<td>Fixed in LA, polymorphic in CARIB</td>
<td>19.0</td>
</tr>
<tr>
<td>Fixed in CARIB, polymorphic in LA</td>
<td>22.9</td>
</tr>
</tbody>
</table>

We identified 135,035 SNPs from the pooled sequence information obtained from all isolates used in this study. This table shows the percentage of the total SNPs that fall into a given category. LA, Louisiana population; CARIB, Florida–Haiti–Yucatan population.
Genes Inside Divergence Islands Have Functions and Patterns of Variation Consistent with Local Adaptation. The difference in latitude between the Louisiana and Caribbean populations suggests that they may have experienced differences in selective forces related to environmental parameters such as day length and average yearly minimum temperature [5.0 °C for Welsh, Louisiana and 13.8 °C for Homestead, Florida (36)]. We sought to investigate whether our candidate genomic islands of divergence between these two populations could harbor genetic factors that are locally adapted.

The first divergence island is on chromosome 3 and contains a pattern of nucleotide variation consistent with independent selective sweeps within each population: an excess of variants segregating at low frequency and reduced $\pi$ (average number of intrapopulation pairwise differences) within both populations, relative to the flanking regions (Fig. 3 and Fig. S6). We find both the Caribbean and Louisiana haplotypes present among the outgroup strains (Fig. 3), but strains from the same locality always have the same haplotype. These facts are consistent with either a history of gene migration among populations or the presence of both haplotypes in the ancestral population, followed by the sweeping of a single haplotype to fixation within populations.

This region contains the genes $plc-1$ (phospholipase C), an MRH4-like mitochondrial DEAD box RNA helicase, and the unnamed gene NCU06247 [inferred to encode an outer mitochondrial membrane protein (37)]. Coincidentally, Gavric et al. (38) observed this same pattern of divergence in $N.\ crassa\ plc-1$, but, lacking the context of the two different populations and the genome-wide sampling presented here, could not explain it. We also found another mitochondrial DEAD box RNA helicase (homolog of the yeast gene MSS116) as one of 12 genes in the Louisiana population that show the signature of positive selection by the McDonald-Kreitman (MK) test (39) (Table S2). We did not expect to find the MRH4-like RNA helicase in this case because the MK test is confounded by the reduced within-population polymorphism in the genomic islands of divergence.

RNA helicases are key factors in the microbial cold response (40), making it tempting to speculate that they are important to Louisiana $N.\ crassa$, which experience minimum temperatures almost 9 °C lower than their Caribbean relatives.

The second divergence island is on chromosome 7 and was fixated between populations (Fig. 3). As with the helicases, it is tempting to speculate that $frq$ is involved in adaptation, in this case related to differences in local photoperiod associated with the 2.4°–10.6° difference in latitude between the Louisiana population and various Caribbean population localities.

Characterizing the Candidate Adaptive Phenotypes. The distributions of these two populations in conjunction with the RNA helicase and major circadian oscillator that we find within these genomic islands of divergence suggest two major environmental factors that may be promoting local adaptation: temperature and day length. Here we have chosen to focus on the response to low temperature. We chose to focus only on low temperature, rather than both low and high temperature, for several reasons. The global distribution of $N.\ crassa$ is mainly tropical, implying that the extension of its range into more temperate Louisiana is a derived condition (45). In addition, there is a 9 °C difference in the mean annual minimum temperature between Welsh, Louisiana and Homestead, Florida, but only a 0.7 °C difference in the mean annual maximum temperature (36). Thus, although winter...
in Louisiana is noticeably cooler than winter in the tropics, the summers are equally warm.

We predicted that individuals from the Louisiana population would exhibit higher fitness in cold temperature relative to individuals from the Caribbean. To test this prediction, we measured the growth rate of 10 randomly chosen individuals from the Louisiana population and 10 from the Caribbean population at 10 °C and 25 °C. For each individual, we calculated its growth rate at 10 °C as a percentage of its growth rate at 25 °C and found, as predicted, that the reduction in growth rate at 10 °C for strains from the Louisiana population is significantly less than that for strains from the Caribbean population, consistent with Louisiana strains exhibiting higher fitness at lower temperatures (P = 0.031; one-sided Mann-Whitney U test; Fig. 4).

To begin to address the potential role in cold adaptation of the candidate genomic islands of divergence identified by our sequence analysis, we used strains from the N. crassa deletion collection (15) to determine whether genes in these islands were involved in low-temperature growth.

Preliminary growth experiments on null mutants of each locus at 10 °C suggested a cold temperature growth defect in deletions of the MRH4-like RNA helicase, the PAC10-like prefoldin subunit, and the unannotated gene NCU06247 (Fig. S8). To control for unlinked lesions introduced during generation of the deletion strains, and to verify reproducibility, we crossed each marked deletion strain to an unmarked tester strain and compared the growth rate of progeny with and without the deletion marker cassette. This experiment confirmed significant growth defects resulting from deletion of either of the two annotated genes but not NCU06247 (Fig. 4 B–D). The importance of the MRH4-like RNA helicase for growth at cold temperature in N. crassa is consistent with work on RNA helicases in many other systems (40, 46, 47), although the relatively modest effect of deleting this locus may be a consequence of functional redundancy among the 18 known helicases in the N. crassa genome, as has been suggested in Arabidopsis (47).

Taken together, our results indicate that the MRH4-like RNA helicase and the PAC10-like prefoldin subunit are critical for wild-type growth in cold temperatures in N. crassa, lending credence to the model that these genomic islands of divergence are the result of adaptation to low temperature. It should be noted, however, that large-scale fluctuations in climate have taken place since the divergence of these populations ≈0.4 Mya (48). Although there have been four major glacial events during this period, at ≈0.4 Mya the planet was in the middle of an interglacial period with an ice volume and surface temperature that is remarkably similar to current levels (48). In addition, although temperatures were cooler in absolute terms during the glaciations, paleontological studies based on pollen and plant microfossils indicate that the relative difference in temperature between the Florida peninsula and that of Louisiana was still present during the last glacial maximum (49). There is no evidence that this most recent glacial period was much more severe than those that preceded it (48), indicating that the Florida/Louisiana temperature difference likely was maintained since the divergence of these two populations.

Future work will be needed to establish the relationship between sequence variants at these loci, cold tolerance, and other environmental parameters, such as day length, that are relevant to the Caribbean and Louisiana populations. It is especially interesting to consider the possibility that the genes in these distinct genomic regions may be interacting in both the response to cold and the circadian rhythm given that the circadian clock of N. crassa exhibits temperature compensation and can be entrained by temperature in addition to light (50).

Conclusion

Here we have illustrated the utility of combining a “reverse ecology” genome-scan approach with functional characterization of the resulting candidate genes to identify ecologically relevant phenotypes in organisms that are difficult to study in nature. The major benefit of this approach, compared with a purely candidate gene approach, is that it provides a relatively unbiased look across the whole genome, allowing for identification of genes whose role in adaptation may not have been expected a priori. As it becomes easier to obtain large amounts of DNA sequence data, this type of approach is becoming increasingly common and will help facilitate the study of ecologically important nonmodel systems.

Although this approach has been demonstrated in other systems (6–10), here it has been used with a microbe, which is where it may prove to be the most useful. It can be difficult to apply this type of approach to populations of nonmodel organisms because it generally needs to be combined with a nearly complete reference genome assembly or an unfinished assembly paired with a genetic map (51). However, compared with microorganisms, fungi have smaller genomes with a lower repeat density, and low-cost, high-quality de novo genome assemblies from short reads have been achieved for both fungi (52) and bacteria (53). These features of microbes suggest that it is feasible to produce a reference genome assembly from a single individual while additionally resequencing many other individuals at low coverage to obtain polymorphism data that can be used for the genome scan. Furthermore, microbes are generally more amenable to genetic transformation, which may aid in the functional characterization of the candidate genes identified in the genome scan.

Materials and Methods

Identification of SNPs. Messenger RNA-Seq reads that did not map uniquely were discarded. Read alignments from each strain were pooled, and SNPs were identified using a Bayesian approach implemented in the program GigaBayes (54). To be included in the final set of high-quality SNPs, a candidate site was required to be biallelic and needed to meet or exceed the following criteria: coverage of five reads per allele, individual base qualities of 10, aggregate base qualities of 40, and Bayesian genotype probability of 0.90. To further reduce the number of potential false positives, singletons were discarded. Sites with missing data (i.e., the allele of one or more individuals was unidentifiable because it did not meet the above criteria) were excluded from analysis. Using these criteria, we found 5,640 genes that

![Fig. 4. Adaptation to cold temperature and functional characterization of genes within divergence islands.](image-url)
had at least one SNP out of the \(n = 9,800\) genotypes in the genome. These 5,640 genotypes had an average of 14.4 SNPs per gene.

### Analysis of Population Demographics

Demographic parameters were estimated from the Louisiana and Caribbean joint allele frequency spectrum using a diffusion-based approach implemented in the program \textit{dadi} (21). To control for the potential misidentification of ancestral states, we fit the model to two additional datasets: one in which we used two outgroups (\textit{N. tetrasperma} \textit{FGSC} \#2508 (23) and \textit{N. discretae} \textit{FGSC} \#8579 (24)) and one in which we applied a correction that is part of the \textit{dadi} package. The results were nearly identical (Fig. S2), and we report the parameters estimated from the uncorrected spectrum.

### Growth Rate Assays

All strains used in the growth rate assays were a mating type. The location of the hyphal front was recorded at regular intervals until it reached the other end of the tube. Each strain was grown in triplicate in constant darkness inside 25 °C and 10 °C incubators. Crosses involving null mutants were made to the \textit{fluffy} mating type tester strain. The \textit{fluffy} strain contains a mutation at a single locus that makes it accumulate and highly fertile (55). All progeny used in growth rate assays were screened to ensure that they produced macroconidia and thus did not have the \textit{fluffy} mutation. See SI Materials and Methods for more details.

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