



Genetic divergence and the number of hybridizing species affect the path to homoploid hybrid speciation

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Edited by David M. Hillis, The University of Texas at Austin, Austin, TX, and approved August 16, 2018 (received for review June 6, 2018)

Hybridization is often maladaptive and in some instances has led to the loss of biodiversity. However, hybridization can also promote speciation, such as during homoploid hybrid speciation, thereby generating biodiversity. Despite examples of homoploid hybrid species, the importance of hybridization as a speciation mechanism is still widely debated, and we lack a general understanding of the conditions most likely to generate homoploid hybrid species. Here we show that the level of genetic divergence between hybridizing species has a large effect on the probability that their hybrids evolve reproductive isolation. We find that populations of hybrids formed by parental species with intermediate levels of divergence were more likely to mate assortatively, and discriminate against their parental species, than those generated from weakly or strongly diverged parental species. Reproductive isolation was also found between hybrid populations, suggesting differential sorting of parental traits across populations. Finally, hybrid populations derived from three species were more likely to evolve reproductive isolation than those derived from two species, supporting arguments that hybridization-supplied genetic diversity can lead to the evolution of novel “adaptive systems” and promote speciation. Our results illustrate when we expect hybridization and admixture to promote hybrid speciation. Whether homoploid hybrid speciation is a common speciation mechanism in general remains an outstanding empirical question.

speciation | hybridization | mating behavior | *Drosophila* | homoploid

Hybridization can result in the “reorganization of adaptive systems” (1) and lead to the formation of new species through the process of hybrid speciation. When the ploidy of hybrid populations and their parental species does not differ, this process is termed homoploid hybrid speciation (HHS). Despite examples of homoploid hybrid species occurring across the tree of life (2–8), there is an ongoing debate in evolutionary biology regarding the importance and prevalence of HHS as a general speciation mechanism (9–13). One of the primary difficulties in determining the prevalence of HHS, given current empirical data, is that it is difficult to ascribe a causative role to hybridization in generating reproductive isolation (RI) between populations of hybrids and their parental species (9).

One approach that can be used to confirm if hybridization has played a role in speciation is to use experiments that test if hybridization and subsequent admixture lead to the evolution of novel traits that are responsible for generating RI. In a seminal study, Greig et al. (14) crossed the fungi *Saccharomyces cerevisiae* and *Saccharomyces paradoxus* and showed that hybrids in the F₂ generation, while almost completely interfertile, show strong RI from their parental species due to karyotypic changes. Similar experiments have been used to show how hybridization can lead to the evolution of novel ecological [e.g., in *Helianthus* sunflowers (8)] or sexual traits [e.g., in *Heliconius* butterflies (4)], thereby contributing to speciation. The studies mentioned above provide experimental evidence that hybridization can lead to the evolution of reproductive isolation between hybrid and non-hybrid lineages; however, we still lack a general understanding of the conditions most likely to lead to this one outcome (of many) of hybridization.

Comparative studies suggest one factor that is likely to affect the evolutionary consequence of hybridization: the amount of genetic divergence between hybridizing taxa. For example, the number and strength of genetic incompatibilities that segregate in hybrid offspring tend to increase as genetic divergence increases between their parental species (15, 16), and the production of phenotypic novelties (i.e., transgressive phenotypes) has also been shown to increase with genetic divergence between hybridizing taxa (17, 18). As pertaining to hybrid species, Chapman and Burke (19) compared levels of genetic divergence between the parental species of 12 homoploid hybrid species and 26 polyploid hybrid species and found that genetic divergence between the parents of homoploid hybrid species was approximately half that of polyploid hybrids, suggesting that the level of genetic divergence between parental species can affect the probability of HHS. Experimental data testing the relationship between genetic divergence and the probability of HHS are nonetheless lacking.

Here we analyze the evolution of behavioral isolation (BI) in 10,260 admixed populations that were produced by crossing 27 unique combinations of parental species of *Drosophila* spanning levels of divergence from 0.01 to 1.23 (Nei's *D*; previously published estimates from ref. 20; *SI Appendix, Table S1*). Our experimental design forced hybridization and admixture between parental species (see *SI Appendix* for details), allowing us to ask how divergence between parental genomes affects the probability

Significance

Hybridization can promote speciation, and examples of putative hybrid species have now been identified across the tree of life. However, we still know little about the conditions that are most conducive to hybrid speciation. We have used experimental evolution in fruit flies (*Drosophila*) to show that both the genetic difference between hybridizing species and the number of hybridizing species affect the probability that hybrids evolve reproductive isolation from their parental species. Our results provide a systematic test of factors that affect homoploid hybrid speciation. Biologists will now be able to test the predictions that our experiments outline in naturally hybridizing species. These types of data hold the potential to greatly increase our understanding of hybridization's role in generating biodiversity.

Author contributions: D.R.M. designed research; A.A.C. and D.R.M. performed research; A.A.C. contributed new reagents/analytic tools; A.A.C. and D.R.M. analyzed data; and A.A.C. and D.R.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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Data deposition: The data reported in this paper are available from the Dryad repository (<https://doi.org/10.5061/dryad.js734c0>).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1809685115/-DCSupplemental.

Published online September 12, 2018.

each admixed population, the strength of assortative mating within each population of hybrids. Assortative mating was stronger when hybrids were generated between more divergent parental species (polynomial regression with binomial error: $F_{1,503} = 4.37$; $P = 0.037$; Fig. 1B); however, this relationship was weak (estimate of $\beta = 0.043$; adjusted $R^2 = 0.7\%$). When we considered admixed populations that showed evidence of assortative mating under a more stringent cutoff of $\alpha = 0.001$, the relationship between parental divergence and the strength of assortative mating was higher ($F_{1,337} = 11.32$; $P = 0.0009$; $\beta = 0.09$; adjusted $R^2 = 3.0\%$; SI Appendix, Fig. S3). Again, these results are qualitatively the same when correcting for phylogenetic nonindependence (SI Appendix, Fig. S8). Therefore, while the greatest proportion of admixed lineages evolved RI when their parental species had moderate levels of genetic divergence, RI tended to be stronger in more divergent parental crosses.

Behavioral Isolation Between Populations of Hybrids Derived from the Same Parental Cross. We next tested if hybrid populations displayed BI from other, independent, hybrid populations derived from the same parental species. We predicted that if hybrid populations are evolving a mixture of different parental preference/display traits used during mate choice, they would discriminate against independent hybrid populations derived from the same parental species. For each of two parental cross types (*D. paulistorum* ‘Orinoco’ × *D. paulistorum* ‘Amazon’ and *D. yakuba* × *D. santomea*) we selected five hybrid populations displaying BI from their parental species and conducted mate choice trials *en masse*, with females given the choice of four male genotypes: the two parental genotypes, hybrid males from their same population, and hybrid males from a second hybrid population, derived from the same cross type. Hybrid females showed evidence of BI from other admixed populations in 19 of the 20 comparisons between hybrid *paulistorum* populations and 17 of the 20 *D. santomea* × *D. yakuba* populations (generalized linear models, Tukey’s contrasts: $P < 0.01$; Fig. 2), while all control experiments resulted in an equal proportion of control males receiving matings (Fig. 2). This result can be explained if populations of hybrids have evolved novel combinations of parental traits/phenotypes, but the specific parental traits differ

among populations of hybrids (see ref. 25 for an analogous genetic process).

Number of Taxa and the Probability That Hybrids Evolve Behavioral Isolation. The results presented above are for hybrid populations that were the result of crossing two parental species. One of the defining features of homoploid hybrid speciation is that reproductive isolation is due to novel genotypes produced through admixture, and verbal arguments that recurrent admixture between divergent lineages can promote hybrid speciation and adaptive radiation have been put forth in the literature [e.g., the syngameon hypothesis (26, 27)]. This supposition predicts that the more genetic diversity present within a population of hybrids, the more likely it is that that population will go on to evolve novel traits (either sexual or ecological). To test this prediction, we generated 400 hybrid populations for each of three unique three-species combinations (SI Appendix) and compared assortative mating behaviors that evolved after 10 generations of admixture in these “triparental” populations to those of “biparental” populations. We found that triparental hybrid populations were more likely to evolve BI from their parental species than their biparental counterparts (generalized linear mixed model: Wald’s χ^2 test on the number of parental species used to generate a hybrid population: $\chi^2 = 6.465$; $P = 0.011$; Fig. 3). This result suggests that, for a given level of divergence between parental species, the evolution of RI in admixed lineages is constrained by levels of genetic variation (e.g., the number of segregating alleles), with higher levels of genetic variation promoting the evolution of RI between hybrid and parental lineages. Taken together, our results illustrate that the probability of homoploid hybrid speciation following a bout of admixture will, in part, be governed by a tension between the amount of hybridization-supplied genetic variation (promoting hybrid speciation) and the proportion of novel genetic combinations that generate incompatibilities (constraining hybrid speciation).

Discussion

Our experiments provide empirical estimates of conditions that are conducive to the evolution of assortative mating between hybrid lineages and their parental species. Specifically, we find that assortative mating is most likely to evolve when hybrids are produced by parental species with intermediate levels of genetic

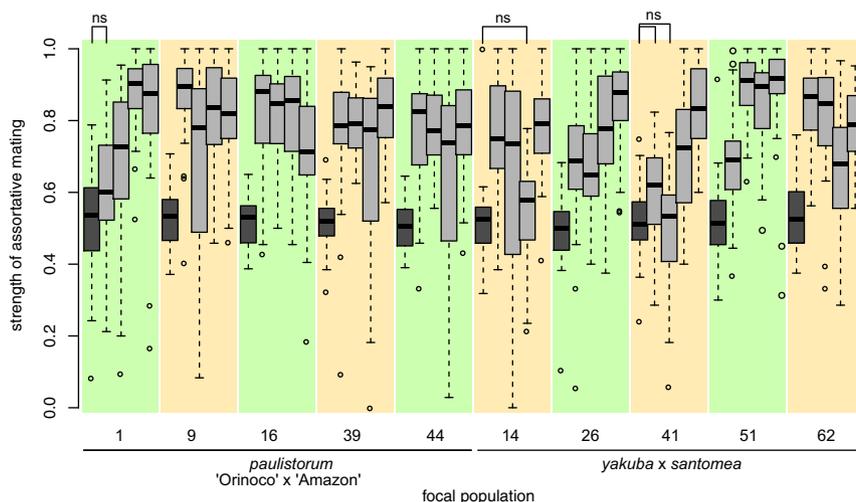


Fig. 2. Hybrid populations that displayed RI from their parental species also showed RI from other hybrid populations. Hybrid populations derived from *D. paulistorum* ‘Orinoco’ × *D. paulistorum* ‘Amazon’ and *D. yakuba* × *D. santomea* showed evidence of RI between other hybrid populations of the same parental type in 36 of 40 pairwise comparisons. Nonsignificant (ns) Tukey’s pairwise contrasts, corrected for multiple comparisons, are indicated with brackets. All comparisons were conducted between the proportion of focal males that mated in control trials (dark-gray boxes) and trials where females were given a choice between males from their same population, a different hybrid population, or males of their parental species (light-gray boxes).

divergence. Using species pairs from the *melanogaster* species subgroup where estimates of genetic divergence as both Nei's D and divergence at synonymous sites (K_s) are available (28), our results predict that hybrids produced by parental species that have diverged at 1.7–9% of synonymous sites will be more likely to evolve RI than those produced by parental species that show either less or more genetic divergence (see *SI Appendix, Fig. S9* for correlation between Nei's D and K_s).

A potential mechanism underlying the evolution of BI in hybrid lineages is that hybrid trait values for traits involved in mate choice are outside the range of parental trait values (i.e., there is transgressive segregation of traits involved in mate choice). Previous studies have demonstrated that the frequency of transgressive segregation in hybrids increases as divergence between parental species increases (17, 18). Our results suggest that “functional transgressive segregation”—that is, transgressive segregation that is not strictly deleterious in nature—will be observed most frequently in hybrids between parental species with intermediate levels of divergence. This explanation is consistent with hybrids between highly divergent parents tending to prefer one parental species over the other (Fig. 1C).

Another, nonmutually exclusive, explanation for the evolution of RI being more likely in hybrids produced by parental species with intermediate genetic divergence is that we expect incompatibilities that exist between strongly diverged parental species to result in strong selection acting against mixed ancestry (*SI Appendix, Fig. S10*). Using divergence as a proxy for the number and strength of genetic incompatibilities (15, 16, 29), selection against mixed ancestry is therefore likely to limit the opportunity for recombinant haplotypes to form, for hybrids to maintain high relative fitness, and for novel phenotypes (such as behavioral preferences) to evolve (30). Indeed, this is a plausible explanation for why we were only able to produce hybrids between two species pairs with Nei's $D > 0.6$ (Fig. 1).

Alternatively, if behavioral traits such as preferences are controlled by multiple groups of interacting loci, hybrid populations could evolve to be a mixture of parental traits. These novel hybrid traits could generate RI between hybrids and their parents. This mechanism predicts that mate choice will be the result of multiple preference/signal traits and, if these traits act in an additive fashion, that RI between admixed and parental lineages will tend to be weaker than between the parental lineages. We did not, however, find support for this in our data (*SI Appendix, Fig. S7*). Future work exploring how ancestry segregates within admixed populations that do versus do not evolve reproductive isolation from their parental species could be used to gain insights into the genetic and phenotypic processes governing HHS. Genomic data would also facilitate tests of the role that structural rearrangements (e.g., inversions) play in homoploid hybrid speciation, a test that is warranted given examples of naturally occurring homoploid hybrid species (2, 3, 8).

Our experimental design allowed us to test the role that divergence between parental species played in the likelihood that their hybrids would evolve reproductive isolation. However, it is worth noting that additional factors are known to play a role in homoploid hybrid speciation. For example, the evolution of novel ecological traits has been shown to play an important role (3, 4, 8). The experiments we have presented in this article were all carried out in a relatively invariant laboratory environment. We also lack meaningful ecological data for the vast majority of the parental species that we used to generate hybrids. Future work testing whether the hybrids of ecologically divergent species are more likely to evolve reproductive isolation than those of ecologically similar species, and/or raising hybrid populations under different environmental conditions, would provide an important experimental test of ecology's role in homoploid hybrid speciation.

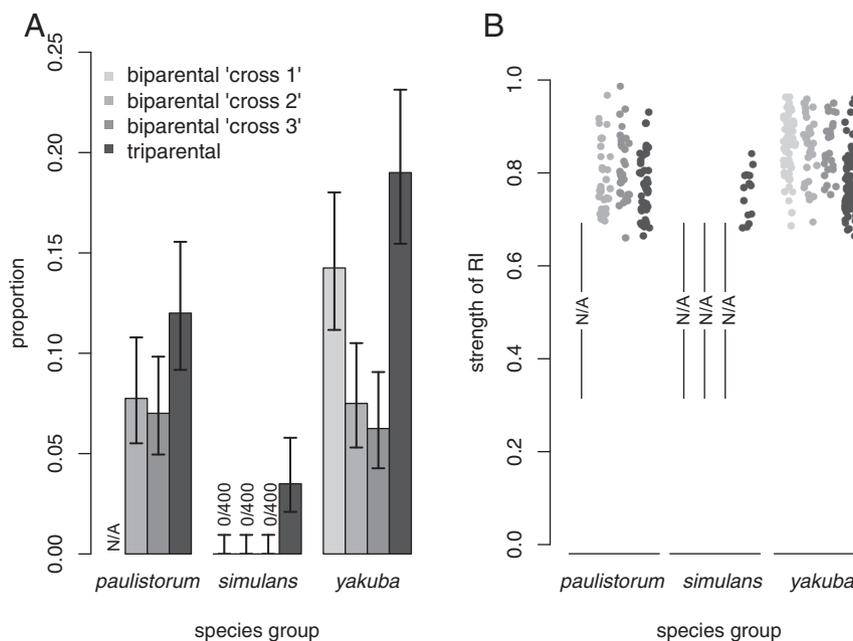


Fig. 3. Hybrid populations derived from three parental species are more likely to evolve behavioral isolation from their parental species. (A) A larger fraction of hybrid populations evolved behavioral isolation from their parental species when they were the result of crossing three parental species compared with two. However, the strength of assortative mating (i.e., reproductive isolation) within populations that did show evidence of evolving behavioral isolation did not differ between biparental or triparental hybrid populations (B). Biparental crosses 1–3 represent the different pairwise combinations of the parental species used to generate hybrid populations that were the result of crossing two species, and triparental hybrid populations were generated by crossing all three of the parental species (*SI Appendix*). The “NA” in A indicates that one of the three possible biparental crosses for the *paulistorum* flies was not conducted.

While a specific test of the mechanism underlying the evolution of reproductive isolation in the hybrid populations that we generated here was outside the scope of our experiment, our results do point to a sweet spot [or “Goldilocks zone” (31)]—in terms of genetic divergence between hybridizing taxa—that will be most conducive to hybrid speciation. Previous theoretical work has shown how HHS, or more specifically, the ability of hybrids to evolve novel and stable recombinant haplotypes, is affected by the form and strength of selection acting on admixed genotypes and the genetic architecture of loci under selection (30–32). These studies provide theoretical support for the idea that hybrid speciation becomes less likely as the number and strength of incompatibilities increase to a level where recombinant genotypes suffer a large selective disadvantage over parental genotypes. As methods designed to detect the timing and amount of admixture continue to be developed (33–37), empirical studies of species and populations in nature can now address questions such as whether there is indeed a “divergence sweet spot” where admixture is most likely to occur.

An important caveat of our experimental design is that we imposed forced hybridization: i.e., parental species were not given a choice of mates and F_1 females could mate only with parental-species males. This design bypasses initial—and potentially strong—reproductive isolation between parental species. Both how mate preferences segregate in natural populations and the geographic context of hybridization should have a large effect on the probability of homoploid hybrid speciation. For example, if hybrids innately mate assortatively (7) or are ecologically differentiated from their parental species (8), it is more likely that they will form a cohesive gene pool and display some level of reproductive isolation from their parental species. By contrast, if hybrids have reduced fitness (e.g., due to incompatibilities) or mate indiscriminately, homoploid hybrid speciation should be less likely. The relative influence of these factors in promoting or constraining homoploid hybrid speciation remains an outstanding empirical question.

Ultimately, studies in natural systems that forge links between admixture and traits that affect reproductive isolation are the only way to test the general prevalence of hybridization as a speciation mechanism. Our results inform where these studies might expect to see hybridization and admixture lead to the production of novel species, a fundamental unit of biodiversity.

Materials and Methods

Species Used to Generate Hybrid Swarms. We chose species to generate hybrid populations after a literature review of reports of a total of 625 previously attempted hybridizations between species of *Drosophila* (20). Our initial screening selected species satisfying three criteria: first, hybridization produces fertile F_1 females in the two reciprocal directions of the cross. The only exception is *D. melanogaster* × *D. simulans* for which we used mutant stocks (38) to produce fertile hybrid females (see below). Second, at least a portion of hybrid males from backcrosses are fertile. These two criteria ensure that we could produce admixed individuals and that populations would not go extinct. Third, all of the species pairs had homologous chromosomes and were not differentiated by neo-sex chromosomes. This last criterion ensures that any potential novel RI truly originated as a product of processes important in homoploid hybrid trait speciation [also termed “recombinational speciation” (27)] and not through segregation of unbalanced chromosomes [akin to chromosomal speciation by monobrachial fusions (ref. 39)]. The resulting 27 species pairs cover the phylogenetic span of all species of *Drosophila* that can hybridize (SI Appendix, Table S1).

Measuring Assortative Mating. While not the only mechanism of RI important for speciation, behavioral isolation that is due to differences in mating preferences is central to the process of speciation (23, 40). We assessed whether assortative mating evolves in hybrid populations as a proxy for evidence of hybrid speciation. To quantify the magnitude of assortative mating (i.e., behavioral isolation) within a given population of hybrids, we collected 100 females and 100 males from a given hybrid population as virgins, housed them in same-sex vials for 4 d, and, on the fourth day,

combined them in a single vial with 100 virgin males from each of the two parental species (n individuals per trial = 400). Before initiating the experiments, male flies were placed on food containing blue, red, or no food coloring overnight. This allowed us to determine the genotype of males following the mate choice assays as the males take up food dye while feeding and the coloration can be observed on and within their body. The group of males receiving red, blue, or no food coloring was randomized across replicates; however, color-dyeing males shows no effect on sexual fitness or preference (41, 42). Groups of flies in these *en masse* mating trials were allowed to choose mates and initiate copulation for 45 min, after which we lightly anesthetized all individuals in the vial with CO_2 gas. This procedure does not separate mating pairs. We then immediately counted the number of each male genotype (based on coloration) that was in the act of mating with a female in a given replicate (as in refs. 41 and 43). These data were used in all subsequent analyses.

Identifying Populations That Displayed Assortative Mating. For each hybrid population, we first determined whether there was evidence of assortative mating using Pearson’s χ^2 tests. Because we were ultimately interested in whether females from a given population of hybrids showed reproductive isolation from both their parental species, we compared the number of hybrid males observed mating with females in a given trial to the number of males from the parental species that received the most matings. This approach excludes information on the number of matings that occurred between females and the parental genotype that received the fewest matings and is conservative because, when the matings are equally divided between parental types, we have less power to detect a significant preference for hybrid genotypes (and assortative mating). In the main text, we focus on results based on considering a hybrid population to display assortative mating using this test and $\alpha = 0.01$. We also repeated analyses with cutoffs of $\alpha = 0.001$ and 0.005 (results presented in SI Appendix, Figs. S2 and S3). For each unique parental cross we used to generate hybrids, we counted the number of hybrid populations with evidence of evolving RI and the number of populations lacking RI. Unless otherwise stated, we carried out statistical analyses on these counts.

The Strength of Reproductive Isolation. We next determined the strength or magnitude of assortative mating for each hybrid population as the number of hybrid males chosen by females divided by the total number of hybrid males and males of the preferred parental species that were chosen by hybrid females. This estimate of RI ranges from 0 to 1 with 0 representing completely disassortative mating (the situation where no hybrid males were chosen as mates), 0.5 representing random mating (i.e., an equal number of hybrid males and the preferred parent species males being chosen), and 1 representing completely assortative mating (the situation where females mated only with hybrid males). Considering only the parental genotype receiving the most matings allows us to focus on the minimum strength of RI, rather than averaging across RI between both parental species (i.e., in some cases hybrid populations would show strong RI with only parental species, but not the other, and we do not consider this case related to homoploid hybrid speciation).

Evolution or Assortative Mating as a Function of Parental Divergence. We modeled the relationship between genetic divergence (as estimated by Nei’s D) and the proportion of hybrid populations that showed evidence of evolving RI using generalized linear models with binomial error terms. We fit nested polynomials of Nei’s D , of increasing degree, starting with a model describing the proportion of hybrid populations evolving RI as a linear function of Nei’s D and then adding a quadratic term, a cubic term, etc. (SI Appendix, Table S6). We compared sequential nested model fits using likelihood ratio tests (LRTs) with the ANOVA function in R. We retained the best-fit model as the model the fit of which was not improved by adding an additional polynomial term.

For hybrid populations that showed evidence of RI from their parental species (based on Pearson’s χ^2 tests described above), we asked whether the strength of assortative mating was related to the level of genetic divergence between their parental species by modeling the strength of RI as a function of Nei’s D . Here, we fit linear models where the strength of RI is the response variable and Nei’s D is the predictor variable. We again fit nested models adding polynomial terms of increasing degree until adding an additional term did not improve the fit of the model (as determined by LRTs) (SI Appendix, Table S7).

We also asked whether the strength of assortative mating that we observed in hybrid populations was correlated with the strength of premating isolation observed between their parental species. We used previously

published estimates of premating isolation between the parental species (20) and tested for a correlation between mean strength of RI in a given hybrid type and the strength of premating isolation between their parental species using Spearman's rank correlation tests as implemented in the COR.TEST function in R.

We used the same approach described above for the intraspecific control experiment; however, none of the 6,000 intraspecific cross populations (400

populations per cross type across 15 different species) showed any evidence of assortative mating (*SI Appendix, Table S5*).

ACKNOWLEDGMENTS. We thank P. Andolfatto, Y. Brandvain, B. Cooper, C. J. Jones, R. Marquez, C. H. Martin, P. Nosil, M. Schumer, two anonymous reviewers, and the members of the D.R.M. laboratory for helpful scientific discussions and comments. This work was supported in part by NIH Award R01GM121750 (to D.R.M.).

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