Genetic Confirmation and Assessment of an Unauthorized Fish Introduction in Parksville Reservoir, Tennessee

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Abstract: In 2001, Tennessee Wildlife Resource Agency biologists sampled what morphologically appeared to be Alabama bass (*Micropterus henshalli*) in Parksville Reservoir (Tennessee River Basin). Alabama bass, which are morphologically similar to spotted bass (*M. punctulatus*), are endemic to the Mobile Basin and had never been previously stocked in Parksville Reservoir. This study sought to confirm the identification of this nonnative fish species in Parksville Reservoir and assess the extent of hybridization with other black bass species within the lake and surrounding water bodies (Chickamauga Reservoir and tributaries). We used five microsatellite loci known to be highly informative for the identification of spotted bass and Alabama bass to assess the taxonomic identity and extent of hybridization for putative Alabama bass samples collected from Parksville Reservoir ($n = 63$) and spotted bass collected from Chickamauga Reservoir and tributaries ($n = 61$). Of the 63 putative Alabama bass collected from Parksville Reservoir, 62 were classified as that species and one as a putative Alabama bass *x* spotted bass F_x hybrid. Two of 61 spotted bass collected from the Chickamauga Reservoir and tributaries were suspected Alabama bass *x* spotted bass F_x hybrids. Our data indicated that current levels of Alabama bass hybridization were low; however, competitive interactions between this non-native species and other black basses have the potential to severely jeopardize other Tennessee fisheries. Our data serve as valuable baseline data in an effort to clearly document the genetic, ecological, and demographic consequences (if any) of this illegal introduction.

Keywords: Alabama spotted bass, hybridization, microsatellite markers, species identification, spotted bass, STRUCTURE.

Journal of the Southeastern Association of Fish and Wildlife Agencies 1:64–69

Some of the highest levels of aquatic species diversity in North America are found in the freshwater systems of the southeastern United States (Lydeard and Mayden 1995, Crandall and Buhay 2008). Unfortunately, an alarming portion of this rich aquatic fauna is threatened or imperiled due to habitat loss or modification, overharvest, and non-native species (Moyle and Leidy 1992, Jelks et al. 2008). Introductions of non-native fishes are homogenizing southeastern aquatic ecosystems at an alarming rate, and represent one of the most prominent threats to biodiversity worldwide (McKinney and Lockwood 2001). Therefore, documenting species invasions and understanding the ecological and genetic mechanisms contributing to the loss of biodiversity are high priorities.

Black bass species comprise valued sport fisheries in Tennessee, with numerous species being highly sought by anglers (Fiss et al. 2004). All but one of the described species of black basses are found in the southeastern United States, four of which inhabit Tennessee reservoirs and streams (smallmouth bass, [*Micropterus dolomieu*]; largemouth bass, [*M. salmoides*]; spotted bass, [*M. punctulatus*]; redeye bass, [*M. coosae*]; Etnier and Starnes 1993). Unfortunately there have been numerous documented cases of non-native black bass introductions resulting in extensive introgressive hybridization with native black bass species (Whitmore 1983, Avise et al. 1997, Pierce and Van Den Avyle 1997, Barwick et al. 2006). One such species is the Alabama bass (*M. henshalli*), that until 2008 was considered a subspecies of the spotted bass (Baker et al. 2008). Although Alabama bass have been introduced outside their native range numerous times (Lee et al. 1980, Pierce and Van Den Avyle 1997, Barwick et al. 2006), they historically had been found only in Tennessee within the Conasauga drainage (Mobile River Basin) where they are endemic (Baker et al. 2008). Beginning in 2001, Tennessee Wildlife Resources Agency (TWRA) biologists started collecting fish that appeared to be Alabama bass in Parksville Reservoir, an impoundment of the Ocoee River, in the Tennessee River Basin. It was unknown whether these fish were in

fact Alabama bass or some hybrid among native *Micropterus* species. Therefore, the objectives of this study were to use molecular methods to confirm the presence of Alabama bass in Parksville Reservoir and survey for evidence of Alabama bass hybridization within Parksville Reservoir and surrounding rivers.

Methods

Study Area

Located in southeastern Tennessee (Polk County), Parksville Reservoir is a small impoundment of 765 ha in surface area that was constructed in 1911. The reservoir is characterized as eutrophic containing warmwater fish species as well as trout from annual stockings. Largemouth bass comprise the majority of black bass species in the reservoir but smallmouth bass and redeye bass also occur in the system.

Tissue Collection and DNA Extraction

During 2012, TWRA biologists collected a tissue sample from each of 63 putative Alabama bass from Parksville Reservoir using standard electrofishing methods. Tissue samples of Alabama bass collected by Alabama Department of Conservation and Natural Resources biologists from Lay Lake, Alabama, ($n = 27$) and Demopolis Reservoir, Alabama, ($n = 33$) were used as our genetically pure Alabama bass reference database. Our reference dataset of genetically pure spotted bass comprised 75 tissue samples collected by TWRA biologists from four localities throughout the Tennessee-Cumberland drainage (J. Percy Priest Reservoir, $n = 17$; Center Hill Reservoir, $n = 19$; Norris Reservoir; $n = 18$, Watauga River, $n = 21$). In an effort to survey the extent of Alabama bass hybridization, TWRA biologists also collected 61 spotted bass tissue samples from Chickamauga Reservoir ($n = 35$) and Hiwassee River ($n = 26$), Tennessee. All tissue samples were preserved in 95% nondenatured ethyl alcohol and archived at the U.S. Fish and Wildlife Service Conservation Genetics Lab in Warm Springs, Georgia. We also incorporated microsatellite data from genetically pure largemouth bass (collected by TWRA from Reelfoot Reservoir, $n = 30$), Florida; largemouth bass (*M. salmoides floridanus*, used as brood stock by Florida Fish and Wildlife Conservation Commission, $n = 30$); smallmouth bass (collected by TWRA from Harpeth River, $n = 30$); and shoal bass (*M. cataractae*, collected by Columbus State University from Chattahoochee River, $n = 27$).

DNA was extracted from a portion of the preserved fin clip using the DNeasy Blood and Tissue kit protocol (QIAGEN, Inc., Valencia, California). We used five microsatellite markers (Msa-06, Msa-10, Msa-22, Msa-27, and Msa-32) known to show allele frequency differences among black bass species (Seyoum et al. 2013). Single polymerase chain reaction (PCR) amplifications were performed in 8 µL reactions containing 30–100 ng uL^{-1} DNA. For Msa-01 and Msa-05 we used PCR reactions consisting of 1 × *Taq* reaction buffer (GoTaq Flexi, Promega), 2.00 mM $MgCl_2$, 0.30 mM of each dNTP, 0.40 uM of each primer, and 0.12 U *Taq* DNA polymerase (GoTaq, Promega, Madison, Wisconsin). Reagents were the same for Msa-31 PCR except we used 2.38 mM $MgCl_2$ and 0.14 U *Taq*; likewise, for Msa-32 we used 2.50 mM $MgCl_2$ and 0.14 U *Taq*. PCR amplifications were conducted using a GeneAMP PCR System 9700 (Applied Biosystem, Inc., Foster City, California). Loci Msa-01 and Msa-05 PCR conditions were an initial denaturation at 94 C (10 min), followed by a touchdown procedure involving 33 cycles and consisting of denaturing (94 C, 30 sec), annealing, and extension (74 C, 30 sec) cycles, where the initial annealing temperature was initiated at 56 C and decreased by 0.2 C per cycle for 30 sec. In contrast, PCR conditions for Msa-31 and Msa-32 were an initial denaturation at 94 C (2 min), then 33 cycles each at 94 C for 30 sec, 58 C for 30 sec and 72 C for 30 sec, followed by an extension step at 72 C for 7 min.

Data Analyses

Prior to electrophoresis, 2 µL of a 1:100 dilution of PCR product was mixed with an 8 µL solution containing 97% formamide and 3% Genescan LIZ 500 size standard (Applied Biosystems, Inc.). Microsatellite reactions were visualized with an ABI 3130 genetic analyzer (Applied Biosystems, Inc.) using fluorescently labeled forward primers and analyzed using GeneMapper software v4.0 (Applied Biosystems, Inc.). Allele frequencies for known Alabama spotted, spotted bass, and Parksville samples were calculated using the computer program GENALEX v. 6 (Peakall and Smouse 2006) and significance ($P < 0.05$) estimated using the genic differentiation test of GENEPOP v. 4.2 (Rousset 2008).

The taxonomic identity of each sample collected from Parksville Reservoir was determined by first assessing the number of groups (K) present in our collected samples (i.e., known Alabama bass from Lay Lake and Demopolis Reservoir; spotted bass from the four Tennessee locations, and putative Alabama bass from Parksville Reservoir). We expected that the number of groups would be two if individuals collected from Parksville were either spotted bass or Alabama bass. If these individuals, however, were neither species, then our sample would be composed of three groups. The number of groups was estimated using a Bayesian-based clustering algorithm implemented in the program STRUCTURE v2.3.3 (Pritchard et al. 2000, Falush et al. 2003). The program STRUCTURE was run with 10 independent replicates for K (i.e., distinct populations or gene pools), with K set from one to six (to account for potential population structure). The burn-in period was 50,000 replicates followed by 500,000 Monte Carlo simulations run under

a model that assumed admixture and correlated allele frequencies. The estimate of *K* was determined by measuring the rate at which the likelihood function changed with increasing *K* (*delta K*; Evanno et al. 2005) using STRUCTURE HARVESTER v.0.6.93 (Earl and vonHoldt 2012). Once *K* was determined, we used the individual assignment patterns produced by STRUCTURE to determine if fish collected from Parksville Reservoir clustered with known Alabama bass or spotted bass. Since there are no known occurrences of spotted bass in Parksville Reservoir, we reran the STRUCTURE analyses with the inclusion of genetically pure largemouth bass to document any potential hybridization between putative Alabama bass and largemouth bass in Parksville Reservoir.

The estimated likelihood that an individual belongs to a given genetic unit is defined as the *q*-value where the higher the *q*-value, the more likely the individual belongs to a given genetic unit (or taxon). We assessed the appropriate *q*-value for identifying an individual as a pure parental taxon using simulations. Using the program HYBRIDLAB v1.0 (Nielsen et al. 2006) and known allele frequencies from Alabama bass and spotted bass, we simulated 1000 Alabama bass, 1000 spotted bass, 1000 Alabama bass *x* spotted bass F_1 hybrids, and 999 backcrosses (333 each of Alabama bass *x* F_1 hybrid, spotted *x* F_1 hybrid, and F_1 hybrid *x* F_1 hybrid). We used STRUCTURE, with $K=2$, to assign simulated hybrids and backcrosses to simulated pure Alabama bass or spotted bass (STRUCTURE settings were as above). In doing so, an expected distribution of *q*-values was created for pure Alabama bass, spotted bass, hybrids, and backcrosses that was compared to the observed values of Alabama bass, spotted bass, Parksville Reservoir samples, and Hiwassee/Chickamauga samples. Observed and expected *q*-value distributions were tested for significance ($P<0.05$) using the chi square test (Sokal and Rohlf 1995)

We also used STRUCTURE to assess the degree of Alabama bass *x* spotted bass hybridization in adjacent Hiwassee River and Chickamauga Reservoir. The analysis included a comparison between spotted bass samples collected in the Hiwassee River and Chickamauga Reservoir to that of six genetically pure reference samples outlined previously. The program STRUCTURE was run with *K* set to a value of six with all other parameters outlined above.

Results

While there were no observed fixed genetic differences between Alabama bass and spotted bass for each microsatellite marker (Table 1), each marker showed significant ($P<0.001$) allele frequency differences. Simulations indicated that a *q*-value ≥76% was appropriate for identifying an individual as a pure parental taxon (Table 2). Using this value, there was a 0.0 and 21.4% chance of falsely assigning an F_1 hybrid or F_x hybrid/backcross (respectively) as a pure parental taxon (Table 2). The observed distribution of *q*-values for Parksville samples and Hiwassee/Chickamauga samples was significantly different (all $P<0.001$) from the expected distribution for a simulated F_1 hybrid or F_x hybrid/backcross population.

Results from STRUCTURE indicated that the number of groups (*K*) represented by the dataset was two when assessed using the *delta K* method (Figure 1). Of the 63 fish collected from Parksville Reservoir, 62 had a *q*-value ≥86% (Table 1) that STRUCTURE grouped with known Alabama bass. The remaining fish was identified as a putative Alabama bass *x* spotted bass hybrid or backcross (q-value = 0.688). We obtained identical results with the inclusion of the largemouth bass dataset indicating that Alabama bass *x* largemouth bass hybrids were absent from our sample. Nine of the 61 putative spotted bass collected from Chickamauga Reservoir and tributaries did not share any ancestry with Alabama bass. Instead, they shared ancestry between largemouth bass and smallmouth bass (n=1), largemouth bass and spotted bass (n=2), largemouth bass and Florida largemouth bass (n=1), or spotted bass and smallmouth bass (n=4). One fish was identified as a pure largemouth bass. Of the remaining 52 fish, 50 had *q*-values ≥76% (46 of these had values ≥90%) indicating that they were pure spotted bass (note that there is a possibility that these fish are F_x hybrids or backcrosses and falsely assigned as pure spotted bass due to using five microsatellite loci; Table 2). Only two individuals from the Chickamauga Reservoir and tributaries were suspected to be of Alabama bass *x* spotted bass hybrid origin having *q*-values of 0.653 and 0.711 (Table 2).

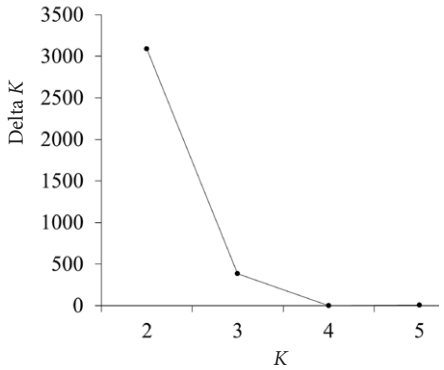

Figure 1. Delta *K* averaged across ten replicate simulations with *K*-values of 1–6. Simulation results indicated that the most plausible value for the number of groups (*K*) represented by putative Alabama bass sampled from Parksville Reservoir, Tennessee, was two as observed by the distinct reduction in delta *K* from *K* of 2 to *K* of 3.

Table 1. Observed allele frequencies of five microsatellite markers for Alabama bass, spotted bass, and putative Alabama bass collected from Parksville Reservoir, Tennessee.

Locus	Allele	Alabama bass	Putative Alabama bass	Spotted bass	Locus	Allele	Alabama bass	Putative Alabama bass	Spotted bass
Msa-06	94	0.039	0.000	0.000		110	0.008	0.105	0.596
	96	0.000	0.024	0.034		112	0.039	0.000	0.053
	98	0.016	0.024	0.867		114	0.008	0.000	0.003
	100	0.016	0.016	0.080		116	0.008	0.000	0.000
	104	0.422	0.234	0.003		118	0.000	0.000	0.121
	106	0.180	0.395	0.009		120	0.000	0.000	0.009
	108	0.289	0.258	0.003		122	0.047	0.024	0.009
	110	0.031	0.048	0.000		124	0.008	0.105	0.047
	112	0.008	0.000	0.000		126	0.000	0.000	0.068
	122	0.000	0.000	0.003		128	0.000	0.008	0.050
						130	0.000	0.089	0.006
Msa-10	120	0.000	0.008	0.000		152	0.000	0.000	0.006
	124	0.000	0.000	0.006					
	126	0.078	0.000	0.006	Msa-32	258	0.000	0.074	0.493
	128	0.000	0.000	0.019		260	0.000	0.000	0.003
	130	0.242	0.310	0.012		262	0.000	0.000	0.014
	132	0.484	0.611	0.025		264	0.008	0.000	0.007
	134	0.031	0.008	0.031		266	0.000	0.009	0.000
	136	0.000	0.000	0.003		270	0.000	0.000	0.293
	138	0.016	0.000	0.000		272	0.105	0.213	0.000
	140	0.070	0.063	0.870		274	0.129	0.000	0.003
	142	0.000	0.000	0.009		276	0.048	0.083	0.007
	144	0.078	0.000	0.000		280	0.048	0.000	0.024
	148	0.000	0.000	0.006		282	0.040	0.000	0.027
	152	0.000	0.000	0.012		286	0.169	0.213	0.000
						288	0.105	0.370	0.003
Msa-22	151	0.817	0.563	0.951		290	0.105	0.019	0.044
	153	0.000	0.000	0.003		292	0.032	0.000	0.037
	159	0.135	0.429	0.015		294	0.048	0.000	0.003
	161	0.024	0.000	0.000		298	0.129	0.000	0.010
	173	0.024	0.000	0.015		302	0.008	0.000	0.024
	177	0.000	0.008	0.015		304	0.024	0.000	0.000
						314	0.000	0.009	0.000
Msa-27	90	0.859	0.669	0.012		334	0.000	0.000	0.003
	92	0.023	0.000	0.000		340	0.000	0.009	0.000
	94	0.000	0.000	0.009		361	0.000	0.000	0.003
	104	0.000	0.000	0.009					

Table 2. Expected and observed *q*-value distributions from STRUCTURE analyses estimated from five microsatellite loci. Expected values are based on 1000 simulated datasets using observed allele frequencies for Alabama bass (Al. bass) or spotted bass (Sp. bass). First generation hybrids and second generation hybrids/backcrosses are denoted F1 and Fx, respectively. Observed values are from sampled Alabama bass, spotted bass, putative Alabama bass from Parksville Reservoir (Parksville), and spotted bass collected from Chickamauga Reservoir and Hiwassee River (Chickamauga). Note that the STRUCTURE analyses identified species other than Alabama bass or spotted bass in the Chickamauga samples; thus, numbers in parentheses represent only the number of spotted bass or putative spotted bass *x* Alabama bass hybrids that were identified from Chickamauga samples (see text for details).

Expected frequency					Observed frequency				Expected frequency					Observed frequency			
q-value	Al. bass	Sp. bass	F1	Fx	Al. bass	Sp. bass	Parksville	Chickamauga	*q*-value	Al. bass	Sp. bass	F1	Fx	Al. bass	Sp. bass	Parksville	Chickamauga
0.5	0	0	674	515	0	0	0	5 (0)	0.76	2	0	0	27	0	0	0	2 (0)
0.52	0	0	42	26	0	0	0	0 (0)	0.78	4	0	0	44	0	0	0	3 (1)
0.54	0	0	89	16	0	0	0	0 (0)	0.8	2	0	0	21	0	1	0	0 (0)
0.56	0	0	84	21	0	0	0	0 (0)	0.82	1	0	0	6	0	0	0	1 (1)
0.58	0	0	31	11	0	0	0	0 (0)	0.84	4	0	0	7	0	0	0	0 (0)
0.6	0	0	32	10	0	0	0	0 (0)	0.86	8	1	0	36	0	0	3	1 (1)
0.62	0	0	27	38	0	0	0	0 (0)	0.88	13	0	0	19	0	0	0	1 (1)
0.64	0	0	7	43	0	0	0	0 (0)	0.9	7	0	0	6	0	1	0	1 (1)
0.66	0	0	5	21	0	0	0	1 (1)	0.92	7	3	0	4	0	0	0	1 (1)
0.68	0	0	4	15	0	0	0	0 (0)	0.94	85	18	0	14	1	1	3	6 (6)
0.7	0	0	2	14	0	0	1	0 (0)	0.96	49	27	0	7	1	5	5	3 (3)
0.72	0	0	2	11	0	0	0	1 (1)	0.98	806	20	0	22	1	12	4	8 (8)
0.74	0	0	1	44	0	0	0	0 (0)	1.00	12	931	0	1	57	55	47	27 (27)

Discussion

Fish collected from Parksville Reservoir were morphologically identified as Alabama bass and genetically consistent with being Alabama bass. The close proximity of Parksville Reservoir to the Conasauga River suggests that Parksville Reservoir Alabama bass were introduced by anglers. Alabama bass tend to grow larger than spotted bass; thus, providing for a potentially better angling experience. For example, the Tennessee state record spotted bass (2.96 kg), which was caught below Parksville Dam, was later invalidated after genetic testing determined it to be an Alabama bass. Recent admixtures between native and non-native congeners could have several outcomes. The introduced taxon can be unsuccessful in the new system, limited by factors such as low propagule pressure or narrow physiological tolerance (Lodge 1993, Marchetti et al. 2004). However, invading and native species can coexist by portioning habitat and sharing resources in a patchy landscape (Schluter 1995, Abrams and Chen 2002, Goclowski et al 2013). Another possibility is that a hybrid swarm could develop, in which introgressed lineages persist with one, both, or neither of the parental taxa (Whitmore 1983, Epifanio and Philipp 2000, Scribner et al. 2001, Barwick et al. 2006). Finally, if competing taxa have similar ecological requirements, then the invading taxon could displace native taxa (Moyer et al. 2005).

Alabama bass are known to be successful invaders of aquatic systems (Lee et al. 1980, Pierce and VanDenAvyle 1997, Barwick et al. 2006) and appear to be abundant and recruiting in Parksville Reservoir. While hybridization is of concern, we are only concerned about elevated incidences of hybridization because low levels of natural hybridization among black basses are common (Whitmore and Hellier 1988, Koppelman 1994). Theoretical and empirical evidence suggest that recently hybridized populations should comprise F_x hybrids at frequencies >99% over a relatively short time period (< five generations; Avise et al. 1997, Epifanio and Philipp 2000), which would translate to a distribution of *q*-values containing a high proportion of estimates ranging from 0.65–0.55 (see expected values from Table 2). Samples from Parksville Reservoir did not meet these expectations. First, the only hybrid detected from Parksville Reservoir was an Alabama bass *x* spotted bass F_x hybrid or backcross; however, this fish was presumably introduced as a hybrid because spotted bass do not occur in Parksville Reservoir. Second, the observed distribution of *q*-values from Parksville Reservoir samples does not meet that of expected for a hybridized or backcrossed population. Our incidence of F_1 hybrids is also in stark contrast to levels found for other black bass introductions (Whitmore 1983, Avise et al. 1997, Pierce and Van Den Avyle 1997). The apparent lack of Alabama bass hybridization in Parksville Reservoir is intriguing because it suggests coexistence with other black bass species under certain conditions. Yet, since the introduction of Alabama bass, largemouth bass have declined precipitously in portions of Parksville Reservoir while Alabama bass have increased in abundance (M. Jolley, TWRA, unpublished data); the majority of Alabama bass have been collected in the lower end of the reservoir whereas largemouth bass have appeared more abundant in the upper end. Thus, monitoring data is indicative of potential habitat partitioning (i.e., local displacement) between Alabama bass and largemouth bass as has been suggested by Greene and Maceina (2000). More monitoring of Parksville Reservoir black bass species will be necessary to determine the eventual outcome of this unauthorized introduction.

The potential for competitive interactions and hybridization between Alabama and other black bass species is a concern because of the unknown ramifications of these interactions, including displacement or extirpation of native taxa. The observation of Alabama bass below Parksville Dam, in the Ocoee River, which empties unrestricted into the Hiawassee River and eventually into the Chickamauga Reservoir of the Tennessee River, heightens this concern because valuable, native smallmouth bass and largemouth bass fisheries could be affected (e.g., Pierce and Van Den Avyle 1997). While we observed only two fish that were of putative hybrid origin between Alabama and spotted bass using a critical *q*-value of 76%, using a more conservative *q*-value of 96% would indicate that 27% (14 of 52 samples) could be of hybrid origin. However, there have been no observed downstream occurrences of Alabama bass other than directly below Parksville Dam and the observed distribution of *q*-values for spotted bass sampled from the Hiawassee and Chickamauga localities was not significantly different than that expected for a pure spotted bass population. Given these observations, we conclude that hybridization between Alabama bass and spotted bass, if occurring, is occurring at a relatively low rate; alternatively, if we rely on the more conservative estimate of *q*, then the population could be in the initial stages of hybridization. Continued monitoring of these populations will help address the ongoing threat of introgressive hybridization.

Often nonnative sport fish introductions are perceived by the general public as providing angling benefits, but the actual ecological consequence(s) of such events are difficult to predict and often have unintended outcomes (Krueger and May 1991, Hickley and Chare 2004, Barwick et al. 2006). We have documented the unauthorized introduction of Alabama bass into the Tennessee River Basin, but the ecological ramifications remain poorly understood. Our data indicated that current levels of Alabama bass hybridization appears low; however, competitive interactions between this non-native species and other black basses have the potential to severely jeopardize other Tennessee fisheries. Our data, along with

annual sampling data from Chickamauga Reservoir and Hiawassee River, will serve as valuable baseline data in an effort to clearly document the genetic, ecological, and demographic consequences (if any) of this illegal introduction so that future human-mediated introductions of this type can be mitigated through management actions and educational outreach.

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