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Molecular marker analysis of European *Setosphaeria turcica* populations

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Abstract

Setosphaeria turcica is the causal agent of northern corn leaf blight, a foliar maize disease of worldwide economic importance. In Europe, its severity increases. To investigate the pathogen's population-genetic structure in central Europe, a total of 80 isolates was sampled in Germany, Switzerland, France, Austria, and Hungary and investigated with 52 random amplified polymorphic DNA (RAPD) markers. The mating type of the isolates was determined in testcrosses. Among the 73 isolates from maize there were 26 different RAPD haplotypes. All isolates with identical haplotype are considered clonemates. The haplotype shared by most members was represented by 22 isolates from Germany, Switzerland, and France, indicating high fitness and substantial migration. Only a single clone had members in both southeastern Austria and southwestern Switzerland, suggesting that the Alps constitute a major barrier for this pathogen. Several haplotypes differed by only one or two RAPD bands from the predominant haplotype and may have arisen by mutation. Few other clonal lineages were detected. The evolution of some haplotypes could not be explained by mutation alone. Sexual recombination may rarely occur. In population samples from Germany, Switzerland, and France, mating type MAT2 was predominating, while most isolates from Austria and Hungary had MAT1. Seven isolates from Johnson grass (*Sorghum halepense*), an alternative host of *S. turcica*, were clonemates and very different in RAPD haplotypes from all isolates collected from maize.

Introduction

The ascomycete *Setosphaeria turcica* Leonard et Suggs with its anamorph *Exserohilum turcicum* (Pass.) Leonard et Suggs causes northern corn leaf blight (NCLB), a serious foliar disease of maize worldwide. The anamorph *Exserohilum turcicum* was first described in Italy 130 years ago (Passerini, 1876; Drechsler, 1923). In South-West France, *S. turcica* appeared at the beginning of this century (Ducomet, 1903; Drechsler, 1923). Epidemics caused by *S. turcica* were observed in northwestern Yugoslavia before 1947 (Spehar and Rojć, 1971). In Europe, the importance of NCLB seems to have increased: In the late eighties it reached epidemic levels first in seed production fields and later in hybrid maize in Austria (Zwatz, 1988; J. Hinterholzer, pers. comm.), as it did in the early nineties in Switzerland (Welz et al., 1996). In Germany NCLB was first found in seed production fields in the Upper Rhine Valley in 1995 (Welz et al., 1996). Most maize varieties in Austria (Zwatz, 1988) and probably other regions are susceptible. Cultivar-specific (*Ht*) virulence is rare in the European *S. turcica* population (Welz and Geiger, 1995).

Among other gramineous species, *S. turcica* can infect Johnson grass (*Sorghum halepense*), which is a serious weed in southern Europe. Johnson grass may serve as an alternate host for part of the pathogen population in Israel (Levy and Pataky, 1992). Physiologic specialization of *S. turcica* towards maize and sorghum was reported from Hawaii (Masias and Bergquist, 1974).

A comparative study of the genetic diversity in *S. turcica* populations in tropical and temperate climates revealed a higher level of diversity in the tropics

Table 1. Geographic origin, designation, number (N), and sampling date of *Setosphaeria turcica* isolates

Country	Region[a]	Location	Isolate codes	N	Date
Germany	URV	Eckartsweier	D-M1.1, D-M1.2, D-M1.3	3	7'94
	URV	Eckartsweier	D-M2.1, D-M2.6, D-M2.9, D-M2.17, D-M2.26, D-M2.30	6	8'95
	URV	Weisweil	D-M3.1, D-M4.2, D-M4.17, D-M4.48	4	8'95
	URV	Kenzingen	D-M5.1, D-M5.8, D-M6.1, D-M6.15, D-M6.30	5	8'95
	URV	Emmendingen	D-M7.1, D-M7.4, D-M7.7, D-M7.10, D-M7.12	5	8'95
	URV	Lahr	D-M10.1	1	11'95
Switzerland	Tessin	Cadenazzo	S-J1.1, S-J1.2, S-J1.3, S-J1.4, S-J1.5, S-J1.6, S-J1.13	7	7'93
	Tessin	Cadenazzo	S-M2.1, S-M2.2, S-M2.3, S-M2.4, S-M2.5, S-M2.6, S-M3.1, S-M3.2, S-M3.3, S-M3.4, S-M3.5, S-M3.6	12	7'93
	URV	Hemishofen	S-M5.1, S-M5.2, S-M5.3	3	9'95
	Tessin	Monda	S-M7.1, S-M7.7, S-M7.27, S-M7.33, S-M7.42, S-M7.49	6	9'95
France	SW-F	Agen	F-M1.1, F-M1.2, F-M2.1, F-M2.2, F-M3.1, F-M3.2	6	9'90
	SW-F	Agen	F-M6.1, F-M6.5, F-M6.6	3	9'92
Austria	Kärnten	Hörzendorf	A-M1.1, A-M2.1, A-M3.1, A-M4.1, A-M5.1	5	9'90
	SM	Gleisdorf	A-M7.1, A-M8.1, A-M10.1	3	9'90
	SM	Spielfeld	A-M16.1, A-M17.1	2	9'90
	SM	Wagendorf	A-M18.1	1	9'90
	SM	Feldbach	A-M19.1	1	9'90
Hungary	Puszta	Szeged	H-M1.2, H-M1.3, H-M1.7, H-M1.8, H-M1.9, H-M1.10, H-M1.11	7	9'96

[a] URV = Upper Rhine Valley, SW-F = Southwestern France, SM = Steiermark.

The first letter encodes the country of origin (D, S, F, A), the second letter the host plant species (M = maize, J = Johnson grass), the first figure the field number within a country, and the figure following the dot encodes the isolate number within a field.

(Borchardt et al., 1998a). Frequent sexual recombination and intense intra-regional migration are likely contributing to this. In the present study, we are focusing on the European population of *S. turcica*. Isolates were sampled from various locations in Germany, Switzerland, France, Austria, and Hungary. They were genotyped with random amplified polymorphic DNA (RAPD) markers and investigated for their mating type. Included were some isolates from Johnson grass, collected in Switzerland. Our specific objectives were (i) to clarify the geographic origin of the recently detected German and Swiss population, (ii) to analyze genetic structures within and among clonal lineages of the fungus, which was feasible because of the large number of loci sampled in the *S. turcica* genome (52 polymorphic loci in the European samples), and (iii) to assess the relatedness of the subpopulations sampled from maize and Johnson grass in Switzerland.

Materials and methods

Fungal isolates

Lesions were sampled from one to nine fields in Germany (D), Switzerland (S), France (F), Austria (A), and Hungary (H) from 1990 to 1996 (Table 1). They originated from both experimental, hybrid-seed production, and commercial fields. One to seven single-conidium isolates per field were cultured and investigated for mating type and RAPD haplotype as described by Borchardt et al. (1998a). A total of 33 – out of 320 prescreened – primers was used including both decamer and simple sequence repeat targeted primers. An amplification profile is given in Figure 1. Reproducibility was excellent due to careful primer screening and use of reference isolates in every PCR run and on every gel (Borchardt et al. 1998a). Fifty-two RAPD bands were polymorphic in the complete set of European isolates investigated, 34 of which detected polymorphisms among isolates from maize. The remaining 18 markers were monomorphic among isolates from maize but distinguished between isolates from maize and Johnson grass. Bands monomorphic in the total set of isolates were not considered.

Data analysis

Multilocus haplotypes, in short haplotypes, are characterized by the presence or absence of bands at all marker loci. Considering that $2^{52} = 4.5 \times 10^{15}$ haplotypes are possible with 52 polymorphic mark-

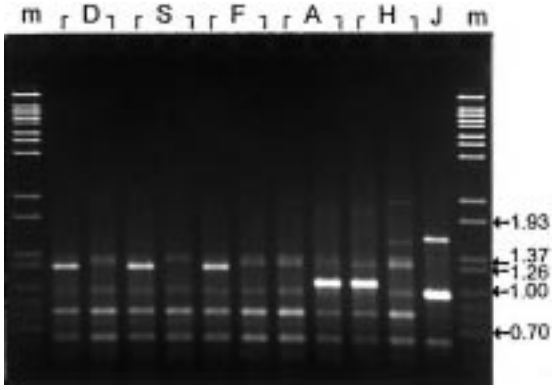

Figure 1. Agarose gel showing RAPD patterns generated by primer 433 with European *S. turcica* isolates. Lanes are designated m for DNA size marker, D, S, F, A, and H for two different isolates each from Germany, Switzerland, France, Austria, and Hungary, and J for an isolate from Johnson grass. Figures to the right designate DNA fragment sizes of the size marker in (kb).

ers, isolates with identical haplotypes are regarded as clonemates.

The Dice similarity coefficient (Sneath and Sokal, 1973) of two isolates x and y was calculated as $S = 2 / (2 + +)$, where , and are the numbers of bands possessed by both isolates, only by x, and only by y, respectively. The software NTSYS-pc (Rohlf, 1993) was used to calculate the unweighted pair-group arithmetic average (UPGMA) algorithm and to develop a dendrogram (procedures SIMQUAL, SAHN and TREE). The matrix-correspondence test (Mantel, 1967) was conducted to compare the similarity matrix with the cophenetic matrix to examine the goodness of fit of the dendrogram to the data (procedures COPH and MXCOMP). The software WINBOOT (Yap and Nelson, 1996) was used to evaluate the robustness of the groupings formed in the dendrogram by bootstrapping. From the binary data set with one isolate per haplotype, WINBOOT reconstructed the phenogram 2000 times by repeated sampling with replacement. The frequency with which a particular group was formed was considered to reflect the reliability of the group.

To investigate the question of putative clonal lineages, individual RAPD band differences between haplotypes were counted and graphically depicted for a subset of haplotypes.

An estimate of genotypic diversity was obtained with the Shannon index, corrected for different sample sizes, as $_S = -\sum_i(_i \ln(_i)) / \ln N$, where g_i is the frequency of the *i*th haplotype in the sample, and N is the sample size. Different H_S-values were compared by a t-test (Poole, 1974), applying appropriate type-I errors via a sequentially rejective Bonferroni procedure (Holm, 1979).

Results

Among the 73 isolates from maize, there were 26 different haplotypes (Figure 2). Of these, 17 haplotypes were represented by only one isolate. Eight haplotypes were detected two to ten times. The most common haplotype members, M4 (Figure 2), was represented by 22 isolates from Germany, Switzerland, and France. This is more than one third of all isolates from these three western European countries. No isolate from east of the Alps, i.e. from Austria or Hungary, was identical to haplotype M4. The dendrogram (Figure 2) shows that haplotypes of most *S. turcica* isolates from the three western countries (M1 to M12) have a higher similarity among themselves than to the Austrian and most Hungarian isolates (M15 to M22). Only four isolates (three haplotypes, M13, M14 and M20) from the three western countries were of high similarity to the eastern isolates. Just one single Swiss isolate had the same haplotype (M20) as some Austrian isolates. Two outgroups are formed by M24 to M26 (three Hungarian isolates) and M23 (a French isolate, which in 61% of all bootstrapped cases clustered together with the Hungarian outgroup haplotypes). The reliability of the dendrogram (Figure 2) is high as the cophenetic correlation is r = 0.99; it is still high (r = 0.93) when the isolates from Johnson grass were disregarded.

Genotypic diversity (H_S) of the whole sample of isolates from maize, regarded as one population, is 0.60, with no significant difference between the eastern (Austria + Hungary: H_S = 0.70) and the western (Germany + Switzerland + France: H_S = 0.51) subpopulations.

The subclustered haplotypes of western European isolates, M1 to M12, were plotted as a minimum spanning tree with single RAPD band differences between the isolates of a branch (Figure 3). Haplotype M4, the one with the most clonemates, assumes a central position. Haplotypes M1 to M9 differ from each other by as many RAPD bands as there are connecting lines between them. Haplotypes M10 to M12 differ from each other by one or two RAPD bands only, but their distance to the cluster around haplotype M4 is inconsistent: Both M10 and M12 differ by 7 bands from both M1 and M6. Further smaller subgroups with dif-

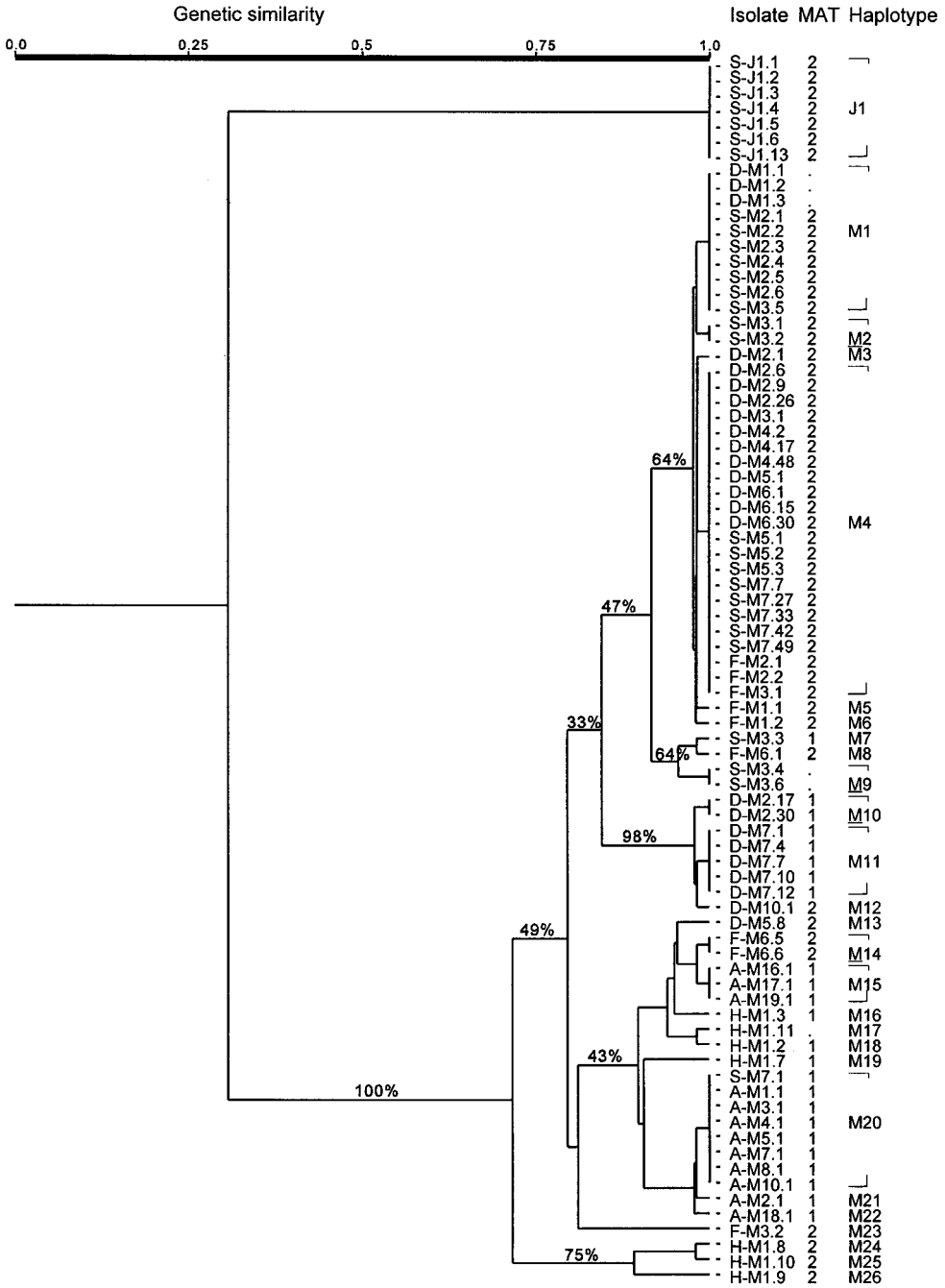

Figure 2. Dendrogram of European *Setosphaeria turcica* isolates obtained by the UPGMA algorithm from pairwise similarities (Dice) of RAPD banding patterns. Values on the branches of the clusters represent the results of bootstrapping analysis (percentage of times the group occurred during 2,000 iterations). MAT = Mating type, '.' = not determined.

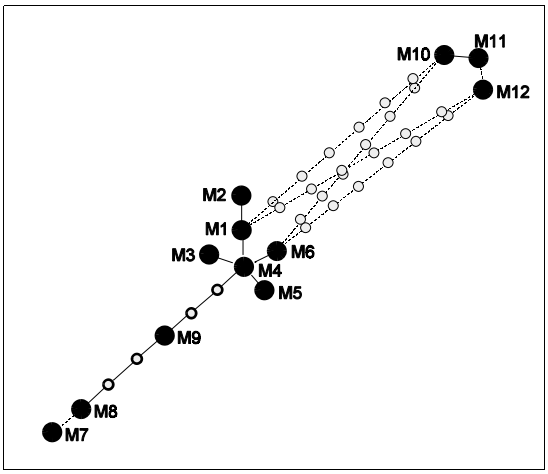

Figure 3. A derivative of a minimum spanning tree with haplotypes of European *S. turcica* isolates. The distance between two adjacent circles corresponds to a single RAPD band difference. Each large circle represents a haplotype found in western Europe (M1 to M12, Figure 2), while the small circles stand for hypothetical haplotypes, which were not detected. The broken lines connecting the small light circles mark possible shortest links between detected haplotypes: Haplotypes M2 and M10, M2 and M12, M6 and M10, and M6 and M12, respectively, all differ in 7 RAPD bands.

ferences in one or two bands between haplotypes and with identical mating type were found (not shown but analogous to Figure 3: M17 - M18; M21 - M20 - M22; and M24 - M25). However, none of them could be linked to other haplotypes or smaller subgroups without forming complicated loops, or without ambiguities. For example, haplotype M16 differs in two bands (and in mating type) from M14 and three other bands from M17, but M14 and M17 differ in three bands, not five.

Both mating types were found among the European *S. turcica* isolates. Isolates with identical haplotype always had the same mating type. The frequencies of MAT1 and MAT2 differed between the three western and the two eastern countries (Table 2). Most isolates from Germany, Switzerland, and France were MAT2. Only the isolates from a single field in Emmendingen (D-M7), two isolates from the experimental station Eckartsweier, and two isolates from Switzerland were MAT1. In contrast, all 12 isolates from Austria, clonemates of four different haplotypes, were MAT1. The Hungarian isolates with highest similarity to the Austrian isolates, haplotypes M16, M18, and M19 had MAT1 as well. Only the three Hungarian outgroup isolates were MAT2. No association between mating type and any RAPD marker was observed.

Table 2. Occurrence of mating types of *S. turcica* in the three western (Germany, Switzerland, France) and the two eastern (Austria, Hungary) European countries

Origin of isolates	Haplotypes with MAT1	MAT2
Germany, Switzerland, France	M7, M10, M11, M20	M1, M2, M3, M4, M5, M6, M8, M12, M13, M14, M23
Sum West:	4	10
Austria, Hungary	M15, M16, M18, M19, M20, M21, M22	M24, M25, M26
Sum East:	7	3

The *Setosphaeria turcica* isolates sampled from Johnson grass in a maize field in Southern Switzerland (Table 1) possessed very different RAPD banding patterns compared to all isolates from maize (Figures 1, 2). One third of all polymorphic bands distinguished between these two groups. All seven isolates from Johnson grass had the same haplotype, designated J1 (Figure 2).

Discussion

Among the isolates from maize, there is a differentiation between the isolates from the eastern (A, H) and western (D, S, F) European countries, as characterized by similarities in marker haplotype and mating type (Figure 2). However, the differentiation was not complete, since some western isolates were most similar to eastern isolates. One Swiss isolate even shared its haplotype (M20) with Austrian isolates. Three Hungarian isolates, regarded as outgroup haplotypes according to their RAPD profiles, had MAT2 which prevailed in the western samples (Figure 2). Moreover, genotypic diversity was not significantly different between the eastern and western samples. Isolation by distance (Wright, 1942) was presumed as an important mechanism forming divergent subpopulations of *S. turcica* on different continents as well as in different regions in Kenya (Borchardt et al., 1998a, 1998b). There is an even larger geographic distance between the French and the Swiss (650 km) or German (690 km) locations than between the Austrian and the Swiss (520 km) or German (550 km) locations. Therefore we conclude that the Alps in their east-to-west extension

may constitute a major barrier to gene flow for this pathogen.

No differences were observed between the population samples from the Tessin and the Upper Rhine Valley, though these two regions are separated by the Alps in north-to-south extension. The two most frequent clones, M1 and M4, consisted of isolates from both the Tessin and the Upper Rhine Valley. Members of M4 were also found in South-West France. Migration must have occurred between these regions, but nothing is known about the time scale. There are also indications of migration over very long distances: The one French outgroup isolate (F-M3.2) beared African alleles, and the Austrian isolates were highly similar to Mexican haplotypes (Borchardt et al., 1998a).

Most haplotypes found in the three western European countries differed only a in single or a few RAPD bands. Figure 3 illustrates the genetic distances among haplotypes M1 to M9 assuming a strictly clonal evolution with mutation being the only source of variability. The base haplotype is likely M4. Out of M4, clones M1, M3, M5, and M6 could have evolved by single-step mutation. A subsequent mutation in M1 may have led to M2. A further putative clonal lineage may be formed by the fairly robust subcluster of M20 to M22 (Figure 2). The putative base clone M4 was represented by 22 isolates, M1 as progenitor of M2 had 10 clonemates in the sample, and the base clone M20 of M21 and M22 had eight clonemates. Obviously, the most recently evolved haplotypes were represented by fewer isolates.

Two intermediate RAPD haplotypes, linking M4 with M9 and M9 with M8, respectively, were not detected. Due to the small sample size they were perhaps missed by chance. Or, M9, M8, and M7 did not evolve by stepwise mutation out of M4, as M7 had the opposite mating type of M8 and M4. Unfortunately, the mating type of M9 could not be determined as no fruiting bodies developed in test crosses. The other outgroup of isolates, M10 to M12, probably did not evolve by a series of mutations out of M4 (Figure 3) because none of the many possible intermediate haplotypes were found. They might even not form a clonal lineage, as M12 differed in mating type from M10 and M11. Clonal lineages were previously reported in populations of *Magnaporthe grisea* (Chen et al., 1995), *Phytophthora infestans* (Fry et al., 1993, Goodwin et al., 1994), and *Sclerotinia sclerotiorum* (Kohli et al., 1992), with mutations occurring at a high enough frequency to be detectable as DNA-fingerprint band changes. In the European *S. turcica* population, clonal lineages are only one aspect of the population structure, not all observations could be explained by this.

Sexual recombination of *S. turcica* probably occurs much less frequently in temperate climates than in the tropics (Borchardt et al., 1998a). Its significance in Europe could not be examined in this study, as not enough haplotypes were available to apply statistical tests. However, rare recombination events may be sufficient to found new clonal lineages. Both mating types were simultanuously detected in three fields (D-M2, S-M3, S-M7), a basic requirement for the sexual stage to occur. Since no RAPD markers were strictly associated with one another or with a mating type, linkage groups are apparently dissolved.

The difference between *Setosphaeria turcica* isolates from Johnson grass and maize is clear-cut. More than one third (18 of 52) of all polymorphic bands generated by the 33 primers routinely used, differentiated between these two groups of isolates. Bootstrapping of the binary data set revealed a complete separation of these two groups (Figure 2). Pseudothecia were formed after pairing with maize-pathogenic tester isolates. In greenhouse tests, the isolates from Johnson grass could not infect maize, or they caused only minute spots or flecks (H.G. Welz, unpubl. data), while the isolates from maize were not tested on Johnson grass. Thus, due to the differences in haplotype and pathogenicity and in contrast to Levy and Pataky (1992), Johnson grass is unlikely to be an alternate host of *S. turcica* in Switzerland. Abadi et al. (1996) also described three *S. turcica* isolates from Johnson grass in Israel that were nonpathogenic to maize and of very different haplotype compared to isolates from maize. But in addition, they described two isolates from Johnson grass, one from Israel and one from Africa, which were pathogenic to maize and had intermediate haplotypes. Heterokaryosis may be one reason for the existence of isolates with combined virulence (Masias and Berquist, 1974).

Further investigations with larger sample sizes are necessary to clarify the genetic structure of populations of *S. turcica* in Europe. Furthermore, the spread of *S. turcica* in Europe should be monitored.

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