Genetic diversity of rice stem borer (*Chilo suppressalis* Walker) from Northern Iran and comparison with other countries

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Abstract

Rice striped stem borer, *Chilo suppressalis* Walker (Lepidoptera: Crambidae) is considered the major pest of rice in Iran. Because of the serious damage on rice in Northern Iran, the present study was conducted to investigate genetic diversity within populations of *C. suppressalis*, from Mazandaran using a template of cytochrome oxidase I gene, 750 bps, (*COI*). Later the haplotypes from Iran were compared with those found in other countries. According to the results of this study, there is very low genetic diversity (two haplotypes) among different populations of this pest in populations of Northern Iran. The genetic similarity and low levels of genetic diversity of these populations suggest that the pest colonization occurred relatively recently and there is high gene flow between these populations of the province. In addition, haplotypes of Mazandaran province are different with those found in other countries. The similarity of Iranian population (Simorgh) with one population from China indicated that China might be the origin of *C. suppresalis*.

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Introduction

Rice is one of the world's most important crops, providing a staple food for nearly half of the global population. It is subject to attack from a range of insect pests that can significantly reduce yields (Arbab 2014). The striped stem borer, *Chilo suppressalis* Walker, (Lepidoptera: Crambidae) is a key pest of rice, which spread from Asia and Pacific to Middle East and Europe (Khan *et al*., 1991). The pole productions of rice in Iran are Mazandaran and Guilan provinces (the Northern Iran). The stem borer is the major pest of rice in Iran especially in Northern Iran. The adults are brownish yellow with silvery scales and a row of 7 or 8 small black dots at the terminal margin of each forewing. The forewings are darker than the hind wings. Damage is created by larval stages. The larvae have a large, shiny brown or orange head, the body color is light brown or pink with five rows of longitudinal stripes that run along the entire length of the body. The larvae cause dead-hearts and whiteheads.

The striped stem borer *C. suppressalis* was collected for the first time from Tonekabon and Ramsar in Mazandaran province. The pest was introduced to Iran in 1973 and then has been widely distributed in all rice fields of Iran (Zibaee *et al*., 2008). There is not detailed information about its settling, however it is supposed that the pest was introduced with straws surrounding citrus entered from Pakistan into the Iran (Okhovat & Vakili, 1997). The pest was limited to Northern Iran for a while but it spread to rice fields of other parts of Iran. Moghaddas and Saiiad-nasiri (1995) reported heavy damages of the pest from other provinces such as Khuzestan, Shiraz, Isfahan and Ilam.

The rice is a main food in Asian people diet, the rice stem borer is a key pest and annually its control costs a lot for the farmers. For these reasons, several research projects have focused on different aspects of the pest as well as the genetic diversity of the stem borer populations in Asia (Ishiguro & Tsuchida, 2006). The *C. suppressalis* has relatively limited dispersal capacity and the adults cannot disperse more than 1-3 km (Khan *et al*. 1991). The pest has discontinuous distributions. Therefore, habitat fragmentation can increase the effect of random genetic drift and lead to local adaptations and population differentiation (Avise 2000). Due to the wide distribution range and low dispersal capacity of this pest, it is expected that some genetic differentiation may occur among different local populations (Liu *et al.* 2013). Different kinds of genetic markers have been used for analysis of diversity in the stem borer populations (Farahpour Haghani *et al*.,

2014). Ishiguro and Tsuchida (2006) investigated the polymorphic microsatellite loci for the rice stem borer. The genetic diversity of *C. suppressalis*, was investigated among 18 Chinese populations by microsatellite, COI, COII, 16S and ND1 (Meng *et al*., 2008). High genetic diversity was found among these populations, this was expected considering the large area of the country and climate variation. Liu *et al*. (2013) also investigated the genetic diversity among seven populations of the stem borer in south of China by six microsatellite, *COI* and *COII* genes. The microsatellite showed polymorphism among the studied populations while the results of two mitochondrial genes indicated no significant difference between these populations and other Chinese populations.

Despite the several research work done on stem borer genetic diversity in other countries such as China, very limited information is available on the genetic variability of stem borer populations in Iran. The only noteworthy research is Farahpour Haghani *et al*. (2014) who investigated the genetic diversity of stem borer among populations from the Guilan province and west of Mazandaran province by RAPD marker. The results of the study indicated that populations from west, east and center of Guilan were different. Also, the populations of Guilan were different from those one of Mazandaran province.

The mitochondrial DNA proved to be very useful, given its haploid nature and maternal inheritance as well as its lack of recombination (Hewitt 2004). This gene was used frequently in genetic analysis of other insects (Chahartaghi-Abnieh 2007; Rajabiyan *et al*., 2015). In present study, the sequences of mtDNAs including the *COI* gene were used for characterization of genetic diversity in stem borer populations in different areas of Mazandaran province (Northern Iran). Furthermore, the sequences of mitochondrial DNA from other countries, recorded in National Center for Biotechnology Information (NCBI) were employed for comparisons.

Material and methods

Larvae of stem borer, *Chilo suppressalis*, were collected from different areas of Mazandaran province in Northern Iran (Table 1). The overwintering larvae were collected after rice harvesting from the infested remained stems in the farms. All samples were preserved in 70% ethanol.

Total genomic DNA was extracted from one individual specimen per each population using the CTAB protocols with some modifications (Nishiguchi *et al*., 2002). DNA was extracted by using a terminal part of individual larva.

The tissues were suspended in pre-warmed (65˚C) CTAB extraction buffer (500 µL) plus -mercaptoethanol (2 µL) and incubated at 65˚C for 1 h and a half. After that 200 µL of chloroform/isoamylalcohol (24:1) solution was added and mixed for 2 min by inverting the microtube. The tubes were placed in centrifuge and the materials were spun 10 min at 13,000 rpm. Then the above phase was transferred to a clean microtube and cold isopropanol (in the same volume) was added. The materials were left for about 24 h to overnight at 20°C and then were spun 10 min at 13,000 rpm. The supernatant was removed carefully and the pellet was washed once or twice with 70% ethanol. 200 µL of ethanol was added and spun at 13,000 for 10 min. The supernatant was removed and the pellet was dried by leaving tubes open under laminar flow hood. The pellet was resuspended in sterile H_2O and stored at 20°C.

The extracted DNA from specimens was amplified using a pair primer, LCO1490 (5 -GGTCAACAAATCATAAAGATATTGG-3') as forward and HCO2198 HCO2198 (5 -TAAACTTCAGGGTGACCAAAAAATCA-3') as reverse (Folmer *et al*., 1994). The COI primer pairs amplify a 750 bps fragment of the Cytochrome Oxidase subunit I gene. Amplifications were performed in 25 µL microtubes containing 0.8 µL $MgCl_2$, 1 µL primers (10 pm/µL), 0.5 µL dNTPs, 2.5 µL PCR buffer, 0.13 Taq Polymerase, 18.57 µL ddH_2O, and 1.5 µL DNA. The amplification program has an initial denaturation step of 5 min at 94˚C, followed by 35 cycles of 60 s in 94°C, 60 s in 52°C, 60 s at 72°C, and a final extension of 5 min at 72 ˚C. Amplification products (5 µL) were visualized after electrophoresis in 2% agarose gels in TAE buffer (24.2 gr Tris, Acetic acid 71 mL, EDTA 10 mL and ddH2O 60 mL) with the 1-log ladder as a molecular weight marker. Purification and sequencing of PCR products for forward primer from individual specimens performed by Takapouzist Company. The accession numbers in GenBank for COI sequences of C. capitata obtained here are KT955018-KT955025.

A Blast search of GenBank sequences using the sequence obtained from the PCR product was conducted through the NCBI website (http://www.ncbi.nlm.gov). Sequences were edited before analysis by BioEdit software (Hall, 2010) and then aligned using Clustal X with defaults parameters (Thompson *et al*., 1997). Phylogenetic trees were constructed using the Maximum Likelihood algorithm in MEGA5 (Tamura *et al*., 2011).

Table 1. Information on sampling sites in Mazandaran province.

Sampling site Locality	Coordinate Longitude (N)	Latitude (E)	Height Sea above level (s.a.l) (m)
Amol	36° 28'	52° 21'	76
Fereydon-Kenar	34° 41'	52° 31'	–21
Simorgh (Kiakola)	36° 34'	54° 49'	–7
Jouybar	36° 33'	54° 47'	–23
Alasht (Savadkooh)	36° 07'	52° 50'	1452
Babol	36° 31'	52° 35'	–9
Noshahr	36° 37'	51° 27'	452
Neka	36° 40'	53° 17'	31

Results

Intraspecific genetic variation was investigated using molecular marker obtained from COI sequences in eight populations of the striped stem borer, *C. suppressalis* from Northern Iran. The total lengths of COI products were 750 bps. The average frequency of bases for COI marker was A=30.1%, T=39.06%, C=15.97% and G=14.87%. The A-T rich sequences were observed in nucleotide composition, which is a pattern that has been repeatedly seen in the mtDNA of insect species (Bajpai & Tewari, 2010).

Phylogenetic trees were constructed using Neighbor Joining method (from 1000 bootstrap replicates) algorithms in MEGA 5 (Tamura *et al*., 2011). A set of striped stem borer mitochondrial DNA sequences including eighteen, available on Genbank, was used in order to compare the Iranian populations with striped stem borer populations from other geographical regions (Figure 1). The species, *Ostrinia nubilalis* (Hübner, 1796) (Lepidoptera: Crambidae) was used as an out-group for construction of phylogenetic trees.

The phylogenetic tree (Figure 1) shows that different populations of the stem borer are similar in COI gene sequences, being placed in one branch separated from the out-group. The population from Simorgh (Kiakola) appears to be a little bit different from the others. The phylogenetic tree also emphasizes a very close genetic similarity among populations of stem borer in Northern Iran, whereas populations of other countries show no similarity with the population in Northern Iran.

Only, the population from Simorgh (Kiakola), shows similarity with one haplotype from China (Figure 1).

Genetic distances were calculated among the eight Iranian populations and some foreign populations using the binary data obtained from primer (Table 2). The genetic distances between northern Iranian populations were 0-0.028. The populations including Amol, Babol, Fereydonkenar, Neka, Savadkooh, Noshahr and Jouybar showed 0 genetic distance and they comprised one haplotype. Very low genetic distance values were found, indicating a high genetic similarity among the derived populations in general. The genetic distance value 0.028 is observed between Simorgh (Kiakola) and other Iranian populations, so this population is the second haplotype. The genetic distance between the Iranian populations and other countries varied from 0.235 to 0.288.

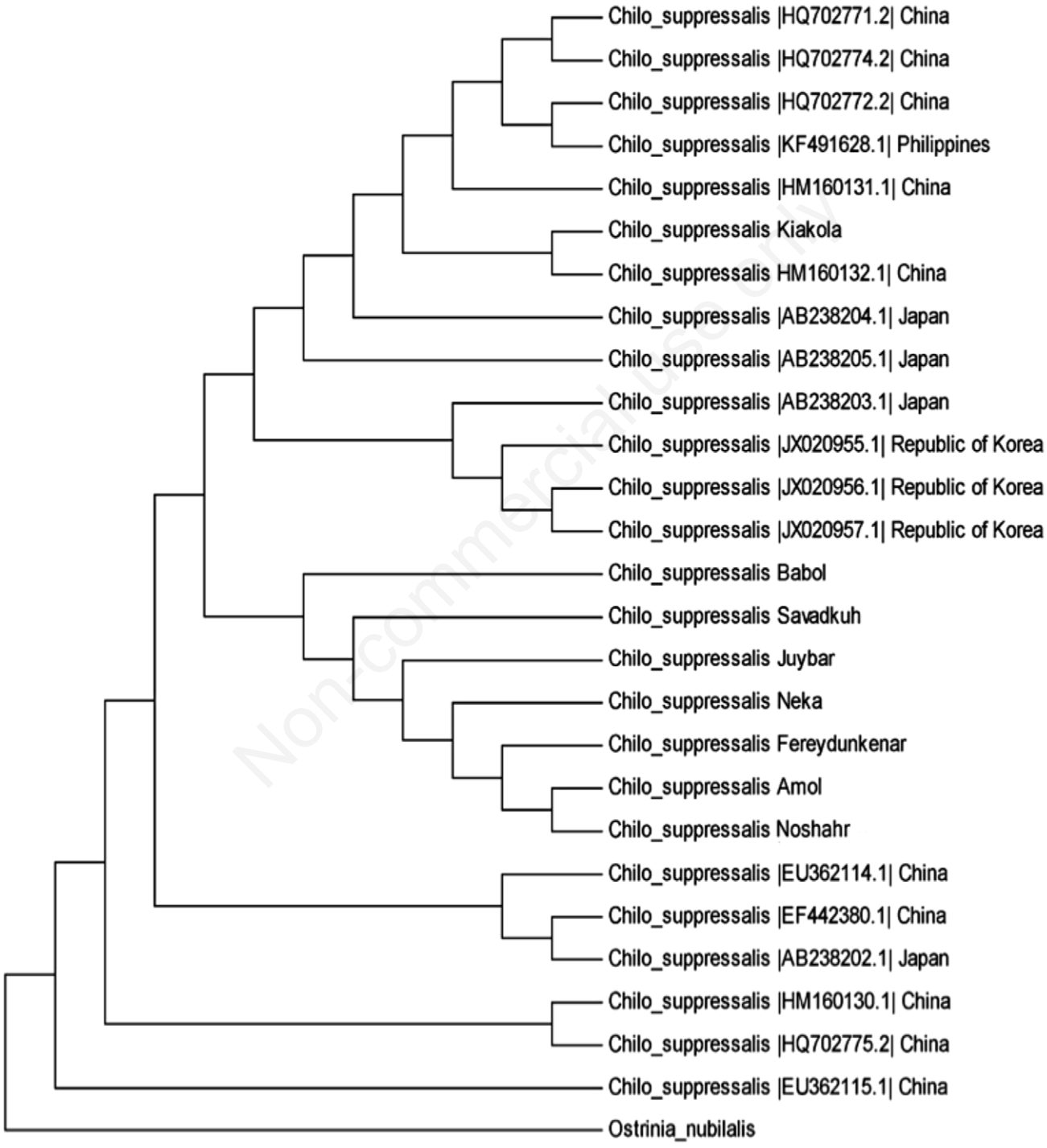

Figure 1. The phylogenetic tree of eight specimens of striped stem borer, *Chilo suppressalis* from Mazandaran province and eighteen other countries for COI, calculated in MEGA5. *Ostrinia nubilalis* used as outgroup.

Table 2. The matrix of genetic distances among the eight specimens of striped stem borer, *Chilo suppressalis* from Mazandaran province and some other countries. *Ostrinia nubilalis* used as outgroup.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1																										
2	0.000																									
3	0.000	0.000																								
4	0.000	0.000	0.000																							
5	0.000	0.000	0.000	0.000																						
6	0.000	0.000	0.000	0.000	0.000																					
7	0.000	0.000	0.000	0.000	0.000	0.000																				
8	0.028	0.028	0.028	0.028	0.028	0.028	0.028																			
9	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.045																		
10	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.032	0.011																	
11	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.045	0.024	0.012																
12	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.032	0.011	0.000	0.012															
13	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.015	0.004	0.015	0.004														
14	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.028	0.023	0.011	0.024	0.011	0.008													
15	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.036	0.024	0.012	0.024	0.012	0.008	0.008												
16	0.019	0.019	0.019	0.019	0.019	0.019	0.019	0.041	0.028	0.015	0.023	0.015	0.019	0.020	0.019											
17	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.024	0.019	0.008	0.019	0.008	0.004	0.004	0.011	0.016										
18	0.004	0.004	0.004	0.004	0.004	0.004	0.004	0.024	0.019	0.008	0.019	0.008	0.004	0.004	0.011	0.016	0.000									
19	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.028	0.023	0.011	0.024	0.011	0.008	0.008	0.015	0.020	0.004	0.004								
20	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.028	0.023	0.011	0.023	0.011	0.008	0.008	0.015	0.019	0.004	0.004	0.008							
21	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.028	0.023	0.011	0.024	0.011	0.008	0.008	0.015	0.020	0.004	0.004	0.008	0.008						
22	0.015	0.015	0.015	0.015	0.015	0.015	0.015	0.045	0.024	0.012	0.024	0.012	0.015	0.024	0.015	0.011	0.019	0.019	0.024	0.023	0.004					
23	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.015	0.004	0.015	0.004	0.000	0.008	0.008	0.019	0.004	0.004	0.008	0.008	0.008	0.015				
24	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.015	0.004	0.015	0.004	0.000	0.008	0.008	0.019	0.004	0.004	0.008	0.008	0.008	0.015	0.000			
25	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.028	0.015	0.004	0.015	0.004	0.000	0.008	0.008	0.019	0.004	0.004	0.008	0.008	0.008	0.015	0.000	0.000		
26	0.008	0.008	0.008	0.008	0.008	0.008	0.008	0.028	0.023	0.011	0.023	0.011	0.008	0.008	0.015	0.019	0.004	0.004	0.008	0.008	0.008	0.023	0.008	0.008	0.008	
27	0.254	0.254	0.254	0.254	0.254	0.254	0.254	0.288	0.274	0.243	0.235	0.243	0.254	0.257	0.266	0.241	0.246	0.246	0.246	0.246	0.257	0.266	0.254	0.254	0.254	0.254

1. Amol, 2. Noshahr, 3. Fereydunkenar, 4. Neka, 5. Jouybar, 6. Savadkooh, 7. Babol, 8. Simorgh, 9. EU362114.1| China, 10. EU362114.1| China, 11. EU362115.1| China, 12. AB238202.1| Japan, 13. AB238203.1| Japan, 14. AB238204.1| Japan, 15. AB238205.1| Japan, 16. HM160130.1| China, 17. HM160131.1| China, 18. HM160132.1| China, 19. HQ702771.2| China, 20. HQ702772.2| China, 21. HQ702774.2| China, 22. HQ702775.2| China, 23. JX020955.1| Republic of Korea, 24. JX020956.1| Republic of Korea, 25. JX020957.1| Republic of Korea, 26. KF491628.1| Philippines, 27. *Ostrinia nubilalis*.

Discussion

The rice striped stem borer, *Chilo suppressalis* Walker, (Lepidoptera: Crambidae) is a key pest of rice in Mazandaran province as a pole of rice production in Iran. Different DNA markers, such as microsatellite, nuclear genes, RAPDs and those derived from mitochondrial DNA, have been used in other infested countries such as China, Japan and South Korea to study genetic variation in striped stem borer populations (Ishiguro & Tsuchida, 2006; Meng, *et al*., 2008; Liu *et al*., 2013). However no research was previously carried out about genetic diversity and relationships of different populations of this pest in Iran, with the exception of the study of genetic diversity of stem borer among populations from the Guilan and west Mazandaran province by RAPD markers (Farahpour Haghani *et al*., 2014). Their results indicated the populations from Guilan were different from those of Mazandaran province.

The genetic distance (Nei, 1972) ranges in local races of a species; low genetic distances among populations of different areas show genetically close relationships among them (Menezes, 1990). The genetic similarities between populations of different areas could help farmers in employing the same successful control methods, since the same race pest could share the same race of natural enemy and this is a key point in for biological control. The low genetic distances found among populations of the stem borer in Mazandaran province from this study indicate that these populations are genetically similar and that colonization in the rice farms by this pest took place once and relatively recently. *C. suppressalis* was introduced to Iran in 1973 by first record from Mazandaran. The short time of pest settlement in Iran did not allow a fixing of genetic variations. The close distance between selected cities, the presence of vast rice fields between the selected sampling locations and finally, the exchanging of the rice products between the cities, caused quick distribution of stem borer in Northern Iran. Additionally, the lack of habitat fragmentation due to similarity of climate, soil composition etc. In the sampling locations have been decreased the chance of genetic divergence. Among Iranian populations, only Simorgh (Kiakola) population showed close relationship with one population from China.

The results of this study indicate that there are only two haplotypes of stem borer in Mazandaran province. Therefore, the colonization of the pest has happened more recently and the pest has not had enough time for genetic divergence. The populations from China showed older colonization than populations from other countries such as Japan, South Korea and Philippine. The populations from Mazandaran clustered separately from other countries indicating that Iranian haplotypes differ from those countries such as China. The similarity of the Iranian population Kiakola with one population from China suggests China as the origin of *C. suppresalis*. High genetic diversities of *C. suppressalis* were observed among the populations of stem borer from China (Meng *et al*., 2008), high diversities could be bound to long-time settlement of the pest in this country, the large extent of China, large distance between examined areas, climate variations and so on. However, the results of two mitochondrial genes (*COI* and *COII*) also indicated no significant genetic diversity among some Chinese populations (Liu *et al*., 2013). The populations from Japan, South Korea and Philippine showed close relationship to each other as well as some haplotypes from China.

Unfortunately, information of pest sequences from many of Asian countries is not available in gene bank. It could be because of high cost of DNA analysis. But, having more information on sequences analysis will provide valuable information about phylogenetic relationship between Asian countries, finding out the origin of the pest, and whether recent colonization or older colonization take place in each country. The information about sequences analysis will also allow for answering some questions relevant to pest management. For example biological control of *C. suppressalis* must consider that the same race of the pest might be share the same natural enemy.

Conclusions

The results of this study indicate no genetic diversity among *C. suppressalis* populations in Northern Iran by COI mitochondrial gene suggesting that pest colonization occurred recently. The populations from Northern Iran (Mazandaran) are separated from the ones from other infested countries such as Japan, South Korea and China. The only observed similarity of Iranian population (Kiakola) was with one population from China indicating that China might be the origin of *C. suppresalis*.

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