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MOLECULAR EVIDENCE FOR NATURAL HYBRIDIZATION IN THE MANGROVE GENUS *AVICENNIA*

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Abstract

Hybridization has been observed in several multi-species genera of mangroves; however, there has been no report on hybridization in *Avicennia* in the Indo-West Pacific region. In this study, we sequenced 5 low-copy nuclear genes of *Avicennia marina*, *A. rumphiana* and 2 individuals of their putative hybrid in the Southeast Asia region to test the hypothesis of natural hybridization between these 2 species. We demonstrated that both putative hybrid individuals possessed two types of sequences at each of the 5 genes, perfectly corresponding to those of *A. marina* and *A. rumphiana*, confirming the hybridization between these 2 *Avicennia* species, and the 2 hybrid individuals are most likely F_1 hybrids. Sequencing of the chloroplast *trnH-psbA* regions indicated that *A. marina* was the maternal parent of the two hybrid individuals.

Key words: Avicennia, chloroplast DNA, Mangroves, Natural hybridization, Nuclear gene.

Introduction

Natural hybridization plays a significant role in plant speciation and evolution (Arnold, 1997; Rieseberg & Carney, 1998; Abbott *et al*., 2013). Mangroves, consisting of 54 species in 20 genera from 16 families, are a unique group of woody plants that grow at the interface between land and sea in tropical and subtropical regions (Tomlinson, 1986; Kathiresan & Bingham, 2001). Interspecific hybridization has been reported in several multi-species genera of mangroves, namely, *Sonneratia* (Duke, 1984, 1994; Tomlinson, 1986; Zhou *et al*., 2005; Qiu *et al*., 2008), *Rhizophora* (Duke & Bunt, 1979; Parani *et al*., 1997; Duke, 2010; Lo, 2010), *Bruguiera* (Sun & Lo, 2011) and *Lumnitzera* (Tomlinson, 1986; Duke, 2006; Guo *et al*., 2011).

Avicennia L. (Avicenniaceae), a genus of mangroves, consists of a small group of tree species and is a major component of mangroves in tropical and subtropical intertidal zones. This genus is widely distributed in both the Indo-West Pacific (IWP) and Atlantic-East Pacific (AEP) regions. There are three species, *A. bicolor*, *A. schaueriana* and *A. germinans*, in the AEP region and five species, namely, *A. marina*, *A. alba*, *A. officinalis*, *A. rumphiana* and *A. integra*, in the IWP region (Duke, 1991). No species of this genus occurs in both regions. A previous study identified ancient introgression between *A. germinans* and *A. bicolor* in the AEP region (Nettel *et al*., 2008). Although multiple species of *Avicennia* coexist in the southeastern Asian region, there have been no reports on natural hybridization among *Avicennia* species to date. During our recent fieldwork on the eastern coasts of Thailand, we found that *A. marina* is dominant in most mangrove stands and sometimes coexists with a rare species, *A. rumphiana*. *Avicennia marina* has ovate-elliptic leaves with a shiny, green upper surface and a dull, pale, finely pubescent undersurface. In contrast, *A. rumphiana* has a dark-green satiny upper surface and a dull, pale russet undersurface. In addition, the fruit of *A. marina* is pale green with a puberulent surface, whereas that of *A. rumphiana* is russet with a woolly tomentose surface. In Chaiya, Surat Thani, Thailand, one of our sampling sites, we found two individuals that were morphologically intermediate between *A. marina* and *A. rumphiana* in their overlapping areas. These two individuals have much broader leaves, a shiny, green upper surface and a pale russet undersurface without any trichomes. Based on the overlapping distribution and intermediate morphology, we suspect that these two individuals are most likely natural hybrids between the two species. However, morphological characters alone are insufficient and sometimes may be misleading in the determination of hybrid status (Morrell & Rieseberg, 1998). Therefore, molecular evidence is needed for the confirmation of hybrid status.

In this study, we sequenced five low-copy nuclear genes of the two morphologically intermediate individuals and their putative parental species, *Avicennia marina* and *A. rumphiana*, to test our hybridization hypothesis. Once the hybrid status was confirmed, we sequenced one intergenic spacer of chloroplast DNA (*trnH-psbA*) from the three taxa to determine the direction of hybridization.

Materials and Methods

Plant materials: We collected *A. marina*, *A. rumphiana* and two individuals of the putative hybrid (CY10 and CY17) in Chaiya, Surat Thani, Thailand. The sampling details are shown in Table 1. To sample as much genetic polymorphisms for each putative parental species as possible, we also collected *A. marina* from Thong Nain Bay, Surat Thani, Thailand, and *A. rumphiana* from Khanom, Nakhon Si Thammarat, Thailand, and Kukup Island, Johor, Malaysia. The leaves from each individual were collected in plastic bags containing silica gel for drying.

Sequencing of five nuclear genes and the chloroplast *trnH-psbA* region: Total genomic DNA was extracted from the dried leaf tissues using the CTAB method (Doyle & Doyle, 1987). Five low-copy nuclear genes (*AJ298992*, *AJ272011*, *Am900316*, *Am900466* and *AU108464*) from an EST database of *A. marina* (www.ncbi.nlm.nih.gov/nucest) were used in this study (Table 2). Primers for the five genes (Table 3) were designed using Primer Premier

5.0 software (Premier Biosoft International, CA, USA). The chloroplast *trnH-psbA* region was amplified using the universal primers *trnH* and *psbA* (Hamilton, 1999). PCR was conducted under the following conditions: 94°C for 4 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1.5 min, and a final extension of 8 min at 72°C. The PCR products were electrophoresed through a 1.0% agarose gel and purified using a Pearl Gel Extraction Kit (Pearl Bio-tech, Guangzhou, China). For each gene, only one single band was amplified from these taxa. The PCR products were directly sequenced using an ABI 3730 DNA sequencer with a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA). To determine the haplotypes of the putative hybrid individuals, we used the pMD18-T Vector System (Takara, Dalian, China) for cloning and then sequenced 6-8 positive clones for each PCR product. All of the sequences have been deposited in GenBank under the accession numbers JX448646-JX448690.

Table 1. Information of sampling sites and sample sizes for the *Avicennia* taxa.

Taxon	Sampling site	Latitude and longitude	Sample size
A. marina	Chaiya, Surat Thani, Thailand	9°25’ N, 99°14’ E	4
	Thong Nain Bay, Surat Thani, Thailand	9°19’ N, 99°45’ E	12
A. rumphiana	Chaiya, Surat Thani, Thailand	9°25’ N, 99°14’ E	1
	Khanom, Nakhon Si Thammarat, Thailand	9°14’ N, 99°52' E	3
	Kukup Island, Johor, Malaysia	1°20’ N, 103°26’ E	12
The putative hybrid	Chaiya, Surat Thani, Thailand	9°25’ N, 99°14’ E	2

Table 2. Sequence variation between *A. marina* and *A. rumphiana* in five low-copy genes and a chloroplast *trnH-psbA* spacer used in this study.

Gene ID	Function/putative function	Fragment length for *A. marina* (bp)[a]	Number of nucleotide substitutions between *A. marina* and *A. rumphiana*[b]
AJ298992	protein kinase	571 (573)	10 (1)
AJ272011	oligouridylate binding protein	590 (590)	17 (0)
Am900316	small nuclear ribonuclear protein F	580 (581)	19 (1)
Am900466	60S ribosomal protein	483 (483)	18 (0)
AU108464	unknown protein	701 (701)	22 (0)
trnH-psbA	a chloroplast intergenic spacer	475 (446)	21 (5)

[a]The number in parentheses is the fragment length for *A. rumphiana*
[b]The number of insertion/deletions is shown in parentheses

Table 3. Primer sequences and annealing temperatures for five nuclear genes and a chloroplast intergenic spacer used in this study.

Gene ID	Primer sequence (5’-3’)	Tm (°C)
AJ298992	F: TCACTCGCAGGGAATTATTCATCG	57
	R: GCTTTCACCGCAGCAGGACA	
AJ272011	F: GGTGGAACTAGCAAGTGGAACATCA	57
	R: TGCACGGGCAGCTTCAGCA	
Am900316	F: ACCGTACCAATCAACCCAAAGC	52
	R: GGCACCCCACGAAGATAAAGA	
Am900466	F: GGAGCAAAGATGTGGTGAA	52
	R: ACCGTACCAATCAACCCAAAGC	
AU108464	F: AATGATTCTAGCAAGTTCCAAATATG	53
	R: TGTGCTGTGACTTTCTTAAAATTCTC	
trnH-psbA	See details in Hamilton, 1999	53

Hamilton, M.B., 1999. Four primer pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Mol. Ecol*. 8, 521–523.

Sequence data analysis: The sequences were aligned with SeqMan software (DNASTAR Inc., Madison, WI, USA). The haplotype network for each gene was constructed using the Network 4.6 program (www.fluxus-engineering.com) with the median-joining method (Bandlet *et al*., 1999).

Results

Sequence analysis of the five nuclear genes in *A. marina, A. rumphiana* and their putative hybrids: The five nuclear genes were directly sequenced from both *A. marina* and *A. rumphiana*. No sequence variation was detected in four of the nuclear genes across all individuals of *A. rumphiana* sampled from three populations, and only one nucleotide variation was observed in *Am900466*. *A. marina* exhibited a slightly higher level of polymorphism than *A. rumphiana*. In *A. marina*, the *Am900316* gene exhibited the highest polymorphism, with three haplotypes being detected. One or two haplotypes were detected for the other four genes. As shown in the haplotype networks (Fig. 1), *A. marina* was well separated from *A. rumphiana*. The variable sites of the five genes in *A. marina*, *A. rumphiana* and their putative hybrids are shown in Tables 4-8. For the five low-copy nuclear genes, both individuals of the putative hybrid possessed two types of sequences, which perfectly corresponded to those of *A. marina* and *A. rumphiana*. For example, for the *AJ298992* gene, the haplotypes of both individuals of the putative hybrid were a combination of haplotype mA1 of *A. marina* and the only haplotype of *A. rumphiana* (Fig. 1A). Over the five nuclear genes, the two putative hybrid individuals differed only for *Am900316*; the alleles corresponding to *A. marina* were mC1 and mC3 (Fig. 1C).

Sequence analysis of the chloroplast *trnH-psbA* region in *A. marina, A. rumphiana* and their putative hybrid: The sequences of the chloroplast *trnH-psbA* region generated from *A. marina* and *A. rumphiana* were 475 bp and 446 bp in length. No variation was found across all individuals within each of the two species, whereas *A. marina* differed from *A. rumphiana* by 21 nucleotide substitutions and 5 indels in this region (Table 9). Both individuals of the putative hybrid had the same *trnH-psbA* sequence as *A. marina*.

Table 4. Variable sites of the *AJ298992* gene in *A. marina*, *A. rumphiana*, and their putative hybrid (CY10 and CY17). Numbers represent the positions of variable sites.

Taxon (haplotype)	Variable site											
	47	201	287	289	322	333	358	360	392	481	505	512
A. marina (mA1)	C	G	A	T	C	G	T	T	G	A	T	--
A. marina (mA2)	C	A	A	T	C	G	T	T	G	A	T	--
CY10	Y	G	R	Y	M	K	Y	Y	K	R	K	AT/--
CY17	Y	G	R	Y	M	K	Y	Y	K	R	K	AT/--
A. rumphiana (rA1)	T	G	G	C	A	T	C	C	T	G	G	AT

Table 5. Variable sites of the *AJ272011* gene in *A. marina*, *A. rumphiana*, and their putative hybrid. Numbers represent the positions of variable sites.

Taxon	Variable site																
	73	86	99	103	125	158	255	308	322	323	418	456	464	465	567	572	585
A. marina (mB1)	C	C	C	A	A	T	A	G	C	G	G	G	C	A	C	T	C
CY10	M	Y	Y	M	R	W	M	S	M	R	R	S	Y	R	Y	Y	Y
CY17	M	Y	Y	M	R	W	M	S	M	R	R	S	Y	R	Y	Y	Y
A. rumphiana (rB1)	A	T	T	C	G	A	C	C	A	A	A	C	T	G	T	C	T

Discussion

Seven varieties were previously recorded for *A. marina*, the most widespread species in this genus. *A. rumphiana* was once treated as a variety of *A. marina* (Moldenke, 1960) and only recently received species rank based on an extensive reassessment of morphological characters and allozyme data (Duke, 1991). In this study, *A. rumphiana* showed deep divergence from *A. marina* with regard to the sequences of five nuclear genes and a chloroplast fragment, also supporting it as a distinct species. For five low-copy nuclear genes, both individuals of the putative hybrid possessed two types of sequences, which perfectly corresponded to those of *A. marina* and *A. rumphiana*. Thus, we provide convincing molecular evidence for natural hybridization in *Avicennia* in the IWP region. Because the hybrid individuals exhibit additivity of the biparental sequences at five nuclear loci, they should be F_1 hybrids ($p<(1/2)^5$ as non-F_1 hybrids, with P referring to the probability). Moreover, the two hybrid individuals show the same chloroplast *trnH-psbA* sequences as *A. marina*, indicating that *A. marina* was the maternal parent of these two hybrid individuals, assuming that chloroplast DNA is also maternally inherited in *Avicennia*.

At least three factors may contribute to hybridization between *A. marina* and *A. rumphiana*. First, the geographic ranges of *A. marina* and *A. rumphiana* largely overlap. *A. marina* is widely distributed throughout the entire IWP region (Duke, 1991), whereas *A. rumphiana* is confined to Malaysia, the Philippines and eastern Indonesia to Australasia (Tomlinson, 1986; Duke, 1991). Therefore, these species overlap almost in the entire range of *A. rumphiana*. The two species show only a slight differentiation in habitat. *A. marina* occurs throughout the tidal range of mangroves, from approximately mean sea level to a high intertidal level (Duke, 1991), and *A. rumphiana* is usually found in the high intertidal zones of downstream estuaries (Robertson & Alongi, 1992). Second, the two species have partially overlapping flowering periods. *A. marina* flowers chiefly from November to December, partially overlapping with that of *A. rumphiana*, which occurs from October and November (Duke, 1991). Third, these species have shared pollinators, with bees frequently being observed on the flowers of *Avicennia* species (Tomlinson, 1986).

Despite frequent hybridization in mangroves, it appears that the hybrids are often simple F_1s, such as the hybrids in *Sonneratia* (Zhou *et al*., 2005; Qiu *et al*., 2008), *Lumnitzera* (Guo *et al*., 2011), *Rhizophora* (Lo, 2010) and *Avicennia* in our study. The phenomenon that most hybrids are restricted to F_1s in mangroves suggests that they should be most likely inviable. However, the possibility of "F_1 dominant hybrid zone" in which F_1s with high viability are maintained by ecological selection (Milne *et al*., 2003; Milne & Abbott, 2008; Zha *et al*., 2010; Pandit *et al*., 2013) cannot be excluded. Hence further investigation will help clarify this issue. Despite the low rates of interspecific hybridization, reproductive isolation between hybridizing species in mangroves may be strengthened by a process called reinforcement as a response to maladaptive hybridization (Dobzhansky, 1937).

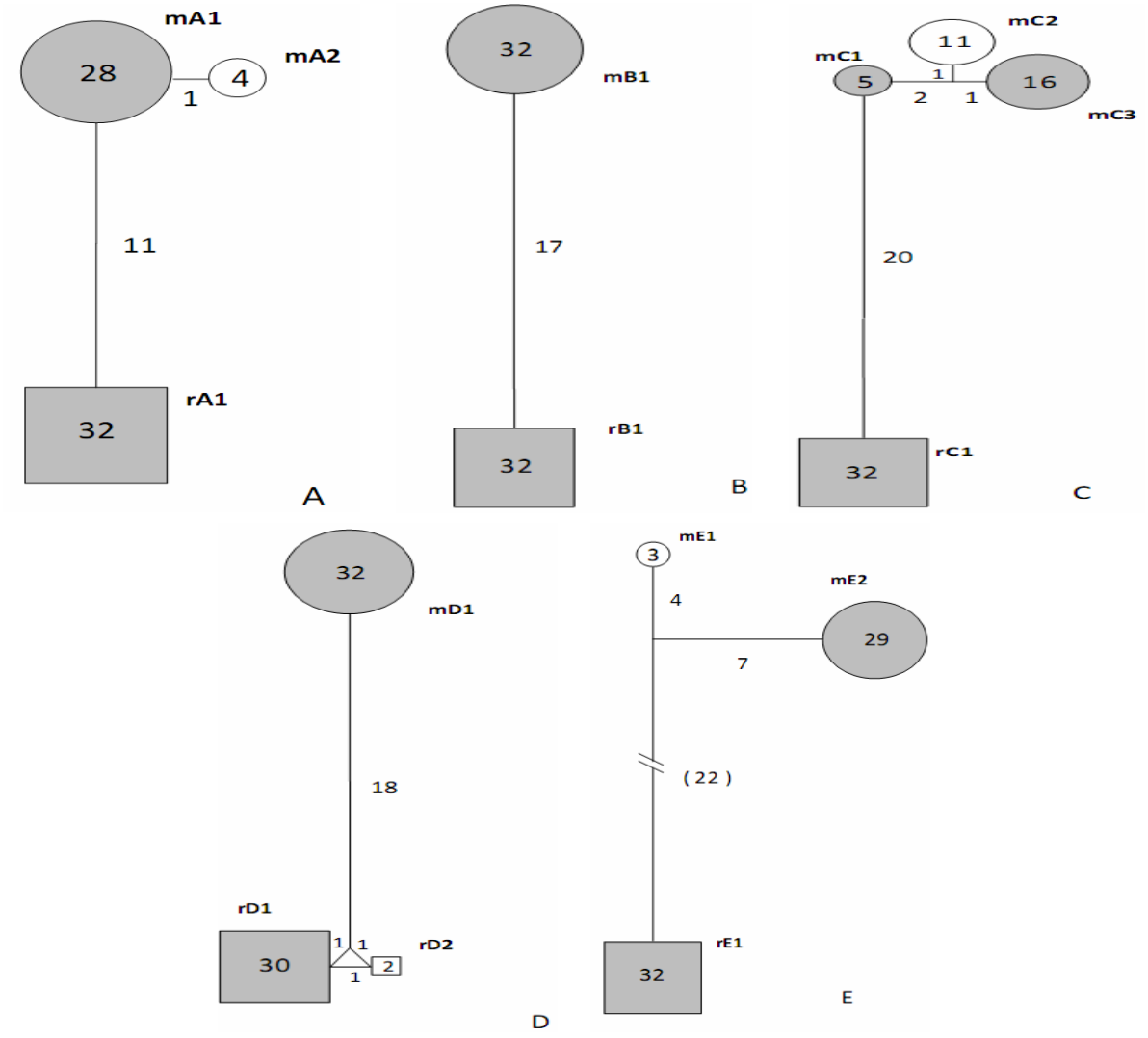

Fig. 1. Haplotype networks of five nuclear genes (A: *AJ298992*; B: *AJ272011*; C: *Am900316*; D: *Am900466*; E: *AU108464*) for *A. marina* and *A. rumphiana*. Circles and squares represent haplotypes of *A. marina* and *A. rumphiana*, respectively, and numbers inside show the frequency of these haplotypes. Shading circles and squares represent the haplotypes involved in hybridization. Haplotypes are named with a lowercase letter, followed by an uppercase letter and a number, representing species, gene and haplotype number, respectively. For example, mA1 means haplotype 1 of gene *AJ298992* in *A. marina.* Numbers close to the solid lines indicate mutational steps between haplotypes.

Table 6. Variable sites of the *Am900316* gene in *A. marina*, *A. rumphiana*, and their putative hybrid. Numbers represent the positions of variable sites.

Taxon	Variable site																							
	9	35	41	55	56	141	153	194	221	222	223	268	285	287	288	317	320	327	329	372	373	438	467	532
A. marina (mC1)	T	G	C	C	G	A	T	C	T	G	G	G	C	C	C	G	T	C	T	A	T	_	A	T
A. marina (mC2)	T	G	C	C	G	A	T	C	T	G	C	A	C	C	C	T	T	C	T	A	T	_	A	T
A. marina (mC3)	T	G	C	C	A	A	T	C	T	G	G	A	C	C	C	T	T	C	T	A	T	_	A	T
CY10	W	K	M	Y	R	M	Y	Y	Y	R	G	R	Y	S	S	K	Y	M	Y	M	Y	A/-	R	K
CY17	W	K	M	Y	G	M	Y	Y	Y	R	G	G	Y	S	S	G	Y	M	Y	M	Y	A/-	R	K
A. rumphiana (rC1)	A	T	A	T	G	C	C	T	C	A	G	G	T	G	G	G	C	A	C	C	C	A	G	G

Table 7. Variable sites of the *Am900466* gene in *A. marina*, *A. rumphiana*, and their putative hybrid. Numbers represent the positions of variable sites.

Taxon	Variable site																		
	37	71	76	85	86	135	152	186	232	236	240	265	324	335	360	387	391	416	466
A. marina (mD1)	G	A	A	T	G	A	G	T	T	A	T	G	A	C	C	C	A	G	T
CY10	R	R	M	Y	S	W	R	Y	W	M	Y	K	W	M	Y	M	M	R	Y
CY17	R	R	M	Y	S	W	R	Y	W	M	Y	K	W	M	Y	M	M	R	Y
A. rumphiana (rD1)	A	G	C	C	C	T	A	C	A	C	C	T	T	A	T	A	C	A	C
A. rumphiana (rD2)	A	G	C	C	C	T	A	C	A	C	C	T	T	T	T	A	C	A	C

Table 8. Variable sites of the *AU108464* gene in *A. marina*, *A. rumphiana*, and their putative hybrid. Numbers represent the positions of variable sites.

Taxon	Variable site																
	22	**44**	**45**	**62**	**63**	**81**	**171**	**182**	**183**	**229**	**231**	**236**	**240**	**245**	**248**	**254**	**259**
A. marina (mE1)	C	T	T	T	A	A	A	T	T	C	G	G	G	T	T	G	A
A. marina (mE2)	C	G	A	T	G	A	A	A	T	C	G	G	C	T	T	G	A
CY10	Y	G	A	Y	R	M	R	W	W	S	R	K	S	K	Y	R	R
CY17	Y	G	A	Y	R	M	R	W	W	S	R	K	S	K	Y	R	R
A. rumphiana (rE1)	T	G	A	G	A	C	G	T	A	G	A	T	G	G	C	A	G

Table 8. (Cont'd.).

Taxon	Variable site															
	291	**333**	**339**	**395**	**398**	**418**	**426**	**441**	**445**	**468**	**487**	**491**	**507**	**599**	**611**	**681**
A. marina (mE1)	T	T	C	T	C	T	G	A	T	T	T	A	A	C	G	G
A. marina (mE2)	T	C	A	T	C	A	T	A	T	G	G	A	A	C	G	G
CY10	Y	Y	M	Y	M	W	T	R	Y	G	K	W	W	Y	S	S
CY17	Y	Y	M	Y	M	W	T	R	Y	G	K	W	W	Y	S	S
A. rumphiana (rE1)	C	T	C	C	A	T	T	G	C	G	T	T	T	T	C	C

Table 9. Variable sites of chloroplast *trnH-psbA* in *A. marina*, *A. rumphiana* and their putative hybrid. Numbers represent the positions of variable sites.

Taxon	Variable site													
	47	83	158	171	183	186	190	203	211	225-227	231	234-245	248
A. marina (mF1)	T	C	G	C	A	G	T	A	C	–	G	GG…GG	G
CY10	T	C	G	C	A	G	T	A	C	–	G	GG…GG	G
CY17	T	C	G	C	A	G	T	A	C	–	G	GG…GG	G
A. rumphiana (rF1)	G	G	C	A	C	T	C	C	A	ACT	A	–	C

Table 9. (Cont'd.).

Taxon	Variable site												
	293	307-325	326	344-345	357	373	381	390	392	397	407	415	421
A. marina (mF1)	G	AT…TT	C	AG	G	C	G	–	A	C	A	A	C
CY10	G	AT…TT	C	AG	G	C	G	–	A	C	A	A	C
CY17	G	AT…TT	C	AG	G	C	G	–	A	C	A	A	C
A. rumphiana (rF1)	A	–	G	–	C	A	T	A	G	T	T	T	A

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