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PHYLOGENETIC RELATIONSHIPS OF *Quercus* SPECIES (FAGACEAE) IN VIETNAM BASED ON MULTIPLEXED INTER SIMPLE SEQUENCE REPEAT GENOTYPING BY SEQUENCING

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ABSTRACT

Quercus is the largest genus of the family Fagaceae in the world and the second-largest in Vietnam, with approximately 50 species. However, the phylogeny study of the *Quercus* genus in Vietnam has yet to be thoroughly explored. In this study, we utilize the genome-wide single-nucleotide polymorphisms (SNPs) data obtained through Multiplexed Inter Simple Sequence Repeat Genotyping by sequencing (MIG-seq) to explore the phylogenetic relationships among *Quercus* species in Vietnam. The results of this study reveal that all *Quercus* species in Vietnam belong to subgenus *Cerris* and the phylogenetic analysis strongly supports the recognition of two infrageneric sections: *Quercus* and *Ilex* section for the Vietnamese *Quercus*. These results also confirm that the monophyly of *Quercus* in Vietnam is strongly supported by both morphological and molecular data. The results of this study also align entirely with previous research, indicating that *Quercus* species in Asia fall under the subgenus *Cerris*, comprising two sections: *section Ilex* and section *Cyclobalanopsis*.

Keywords: Flora, MIG-seq, NGS, Phylogeny, Oak.

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INTRODUCTION

Quercus L. (Oak) is the biggest genus in the family Fagaceae and contains more than 500 species worldwide. Species diversity is highest in the Southeast Asia tropical montane forests and temperate deciduous forests in East Asia, Europe, and North America and desert scrubs in the Mediterranean (Nixon, 1993; Hubert et al., 2014; Valencia-A et al., 2016). *Quercus* species are dominant trees in evergreen broad-leaved forests and play a significant economic, timber, and ecological role globally (Nixon, 2006; Hubert et al., 2014).

Until now, numerous studies on the phylogenetic relationships between species of the genus *Quercus* have been conducted worldwide. Of which, the infrageneric classification has been carried out by two nuclear-encoded molecular markers, namely, the widely utilized ITS region of the 35S rDNA cistron and a 2415 bp long fragment of the CRABS CLAW gene (CRC). Recognition of five or six major groups has been achieved through these markers, as documented by Manos et al. (2001), Oh & Manos (2008), and Denk & Grimm (2010). These identified groups partially coincide with the consensus reached by the morphological classification systems proposed by Camus (1936–1954), Schwarz (1936), and Menitsky (2005), as reviewed by Denk & Grimm (2010). In recent times, various gene markers (Hubert et al., 2014; Simeone et al., 2016), RAD-seq (Hipp et al., 2014; Cavender-Bares et al., 2015; Fitz-Gibbon et al., 2017; McVay et al., 2017) have proven effective for uncovering phylogenetic relationships within the genus *Quercus* and among closely related species. Until now, previous phylogenetic studies on *Quercus* have rarely included species distributed in Vietnam, particularly the endemic ones.

Vietnam is one of the biodiversity hotspots in the world with approximately 50 species of *Quercus*. The previous studies of this genus mainly focused on the taxonomic work, utilizing both morphological and molecular evidence (Camus, 1936–1954; Ho, 2003; Ban, 2005; Li et al., 2016; Binh et al., 2018a, b, c; Binh et al., 2021., Ngoc et al., 2022a, b). Those studies confirmed the species diversity of the Vietnamese Oak. In recent years, researchers have also used both DNA barcodes of classic and next-generation sequences to clarify the taxonomic status and relationships among the closely related species, as well as describe new taxa in Vietnam (Binh et al., 2018a; Ngoc et al., 2022a, b).

Multiplexed ISSR Genotyping-by-sequencing (MIG-seq) is one method for building next-generation sequencing libraries with PCR-based procedures without restriction enzyme digestion steps. MIG-seq is a powerful, simple, quick, cost-effective genotyping procedure, and can be widely applicable to a wide range of DNA quality and quantities (Suyama & Matsuki, 2015; Binh et al., 2018a; Ngoc et al., 2021; Ngoc et al., 2022).

In this study, we utilize the genome-wide SNPs data obtained through MIG-seq to investigate the phylogenetic relationships among *Quercus* species in Vietnam.

MATERIALS AND METHODS

Taxon sampling and morphological identification

In this study, we used a part of the sample from our previous studies (Binh et al., 2018a, b, c; Binh et al., 2021; Ngoc et al., 2022a, b) and 39 new accessions from this study. A total of 108 samples representing 50 of the Vietnamese *Quercus* species were included in the analyses. Species identifications (Table 1) were made based on carefully comparing their morphological traits with the available type specimens or authentic specimens of all *Quercus* species described from Vietnam, China, Cambodia, Laos, and Thailand. We accomplished this by visiting the herbaria HN, P, VNM, FU, and DLU, and utilizing digital images of specimens on JSTOR Global Plants (https://plants.jstor.org/) and the websites of various herbaria. Additionally, we reviewed the original description of each species. Additionally, seven accessions of six species

of *Lithocarpus* genus were used as outgroups including *Lithocarpus obovatifolius*, *Lithocarpus vinhensis*, *Lithocarpus longipedicellatus*, *Lithocarpus dahuoaiensis*, *Lithocarpus vuquangensis*, and *Lithocarpus hongiaoensis*.

Table 1. List of voucher specimens that were used in this study

Species	Specimens ID	Localities
Quercus poilanei	*QC34, QC44, QC77, QC78, V1895,V339, V703, V1990, V2043 V2907, V2986, V3113*	Bidoup, Nui Ba NP, Lam Dong province; Hon Ba NR, Khanh Hoa province; Bach Ma NP, Thua Thien Hue province; Ba Na NR, Da Nang City
Quercus braianensis	*V3219, V4034, QC33, QC46, QC47, QC48,QC89, V6077*	Bidoup, Nui Ba NP, Lam Dong province; Ngoc Linh NR, Kon Tum province
Quercus kerrii	*QC76*	Ngoc Linh NR, Kon Tum province
Quercus helferiana	*QC64, QC65, V3244*	Bidoup, Nui Ba NP, Lam Dong province
Quercus austrocochinchinensis	*QC83, QC86*	Son Tra, Da Nang City
Quercus bidoupensis	*QC29, QC72, QC30, V3202, V10069*	Bidoup – Nui Ba NP, Lam Dong province
Quercus sp1.	*V6618*	Ngoc Linh NR, Kon Tum province
Quercus sp2.	*V6597*	Ngoc Linh NR, Kon Tum province
Quercus chapaensis	*V5101*	Hoang Lien NR, Lao Cai province
Quercus sessilifolia	*V5112, V5047*	Hoang Lien NR, Lao Cai province
Quercus lineata	*V6028, V7502, V7499*	Cuc Phuong NP, Ninh Binh province
Quercus augustini	*QC166*	Ba Na NR, Da Nang City
Quercus sp3.	*V4365, V9824, V10170*	Bidoup, Nui Ba NP, Lam Dong province
Quercus annulata	*V4730, QC162*	Hoang Lien NP, Lao Cai province
Quercus djiringensis	*V4309, V5537, V5538*	Bidoup, Nui Ba NP, Lam Dong province
Quercus chevalieri	*V6448*	Ngoc Linh NR, Kon Tum province
Quercus macrocalyx	*QC110, QC123, QC149, V5776, V6457*	Bach Ma NP, Thua Thien Hue province; Vu Quang NP, Ha Tinh province; Ngoc Linh NR, Kon Tum province
Quercus auricoma	*V3135*	Son Tra, Da Nang City
Quercus sontraensis	*QC201, V3138, V6965*	Son Tra, Da Nang City
Quercus baniensis	*V3089, V6922*	Ba Na NR, Da Nang City
Quercus sp4.	*V3042*	Hai Van Pass, Da Nang City
Quercus bella	*QC07, V6031, V6038, V6044*	Ba Vi NP, Ha Noi Capital
Quercus disciformis	*V6052, V6053, V6058*	Ba Vi NP, Ha Noi Capital
Quercus quangtriensis	*QC113*	Vu Quang NP, Ha Tinh province
Quercus chrysocalyx	*V3289*	Vu Quang NP, Ha Tinh province

Species	Specimens ID	Localities
Quercus platycalyx	*V6065*	Vu Quang NP, Ha Tinh province
Quercus xuanlienensis	*QC10*	Xuan Lien NR, Thanh Hoa province
Quercus sp5.	*V5724, V5927*	Vu Quang NP, Ha Tinh province
Quercus edithiae	*QC109*	Vu Quang NP, Ha Tinh province
Quercus bambusifolia	*QC99, QC101, QC102, V3587*	Vu Quang NP, Ha Tinh province
Quercus thorelii	*QC106*	Vu Quang NP, Ha Tinh province
Quercus xanthoclada	*V3581, V3718*	Vu Quang NP, Ha Tinh province
Quercus langbianensis	*QC26, QC49, QC58, QC71, V3962, V4165, V4166, V4398, V4465, V10061, V10074*	Bidoup, Nui Ba NP, Lam Dong province
Quercus donnaiensis	*V3208*	Cong Troi, Lam Dong province
Quercus sp6.	*V6136*	Ngoc Linh NR, Kon Tum province
Quercus baolamensis	*QC87*	B40 Pass, Lam Dong province
Quercus honbaensis	*V744, V1200, V1378, V1548, V1662*	Hon Ba NR, Khanh Hoa province
Quercus blaoensis	*V1366*	Hon Ba NR, Khanh Hoa province
Quercus sp7.	*V4285*	Bidoup, Nui Ba NP, Lam Dong province
Quercus setulosa	*QC12*	Duc Trong, Lam Dong province
Quercus trungkhanhensis	*V6066, V7501*	Cao Vit Gibbon, Cao Bang province
Quercus lanata	*QC35, QC36*	Da Lat, Lam Dong province
Lithocarpus obovatifolius	*V2983*	Bach Ma NP, Thua Thien Hue province
Lithocarpus vinhensis	*V3787*	Vu Quang NP, Ha Tinh province
Lithocarpus longipedicellatus	*V3813*	Vu Quang NP, Ha Tinh province
Lithocarpus dahuoaiensis	*V3194, V5404*	Chuoi Pass, Dahuoai, Lam Dong province
Lithocarpus vuquangensis	*V5743*	Vu Quang NP, Ha Tinh province
Lithocarpus hongiaoensis	*V3235*	Bidoup, Nui Ba NP, Lam Dong province

Notes: NP: National Park; NR: Nature Reserve.

DNA extraction, PCR, and sequencing

The DNA from each sample was extracted using the cetyltrimethylammonium bromide (CTAB) method (Doyle & Doyle, 1987) from silica gel-dried leaves. For this study, we followed the modifications outlined by Toyama et al. (2015). Initially, the dried leaf material was finely powdered using the QIAGEN Tissue Lyser and subsequently subjected to three washes in a 1 mL buffer containing 0.1 M HEPES (pH 8.0), 2% mercapto-ethanol, 1% PVP, and 0.05 M ascorbic acid. To assess the quality and quantity of the extracted DNA, we employed a NanoDrop (Thermo Scientific) test.

The procedures of MIG-seq include two PCR reactions following the protocol of Suyama & Matsuki (2015) with minor

modifications as described in the previously published literature (Suyama et al., 2021; Binh et al., 2018a; Ngoc et al., 2021). The ISSR regions from genomic DNA were amplified with MIG-seq primer set-1 (Appendix) during the 1st PCR reaction with the given condition as described in Suyama Matsuki (2015). The product of 1st PCR was checked by a Microchip Electrophoresis System (MultiNA, Shimadzu) with the DNA-2500 Reagent Kit (Shimadzu). Before running the 2nd PCR reaction, the 1st PCR product was diluted 50 times with deionized water. Then, those products were used as the template for amplifying with indexed primer according to the given protocol that had been previously published (Suyama & Matsuki, 2015; Suyama et al., 2021; Ngoc et al., 2021). In the next step, all PCR products were pooled as a single mixture library for purification and size selection. Finally, approximately 10 pM of libraries were used for sequencing with Illumina MiSeq Sequencer after measuring the concentration.

Phylogenetic analyses

Before constructing the phylogenetic tree, the Trimmomatic 0.39 and Stacks 2.41 software (Bolger et al., 2014; Catchen et al., 2013; Rochette et al., 2019) were used for quality control of MIG-seq data and de novo SNP discovery, respectively. Then, the genome-wide SNPs data set was used to infer the maximum likelihood phylogenetic tree using RAxML version 8.2.4 (Stamatakis, 2014) with the GTR + G nucleotide substitution model as determined by jMrModeltest 2.1.10 (Darriba et al., 2012). To evaluate the topological reliability of the phylogenetic tree, 1000 bootstrap replicates were performed.

RESULTS AND DISCUSSION

The maximum likelihood (ML) tree based on MIG-seq genome-wide SNPs is highly resolved and reveals two main clades, including the outgroup with seven accessions of the *Lithocarpus* genus and 108 accessions of the *Quercus* genus. All of *Quercus* samples belong to subgenus *Cerris* and the monophyly of *Quercus* is strongly supported by the highest bootstrap values (100%) (Fig. 1).

The phylogenetic tree of the genus *Quercus* in Vietnam is divided into two major clades, corresponding to the *Ilex* section and the *Cyclobalanopsis* section. A 100% bootstrap value supports both of those sections. Section *Ilex* includes two samples of *Quercus lanata*, two samples of *Quercus trungkhanhensis*, and one sample of *Quercus setulosa*. While section *Cyclobalanopsis* is divided into three groups: *Cyclo.* 1, *Cyclo.* 2, and *Cyclo.* 3. These groups are supported by an absolute bootstrap value of 100% (Fig. 1).

The group *Cyclo*. 1 consists of only one accession of *Quercus* (V4285), and the phylogenetic tree showed that it is isolated from the rest of section *Cyclobalanopsis* (Fig. 1). The morphological characteristics of this accession do not resemble any previously described species of the section *Cyclobalanopsis.*

The group *Cyclo*. 2 comprising 76 accessions of the section *Cyclobalanopsis* and was supported by the highest bootstrap value (100%). Both morphological and molecular data strongly support the monophyly of this group. In the phylogenetic tree, this group showed the highest level of bootstrap value of 100% and included 34 spp. of the section *Cyclobalanopsis* and those identified are *Q. honbaensis, Q. blaoensis, Q. baolamensis, Quercus* sp.6, *Q. dongnaiensis, Q. langbianensis, Q. xanhthoclada, Q. thoreilii, Q. bambusifolia, Quercus* sp. 5, *Q. edithiae, Q. xuanlienensis, Q. platycalyx, Q. chrysocalyx, Q. quangtriensis, Q. disciformis, Q. bella, Quercus* sp. 4, *Q. baniensis, Q. sontraensis, Q. auricoma, Q. macrocalys, Q. chevalieri, Q. djiringensis, Q. annulata, Quercus* sp.3, *Q. augustini, Q. lineata, Q. sessilifolia, Q. chapaensis, Quercus* sp. 2, *Quercus* sp.1, and *Q. bidoupensis.* In this group, nine samples do not resemble any previously known species of the section *Cyclobalanopsis* as well as the genus *Quercus,* but there is not enough evidence to confirm their identification. In this study,

based on morphological observation we stated those samples as *Quercus* sp.1 (V6618), *Quercus* sp.2 (V6597), *Quercus* sp.3 (V9284, V4365, V10170), *Quercus* sp.4 (V3042), *Quercus* sp.5 (V5724, V5927), and *Quercus* sp.6 (V6136).

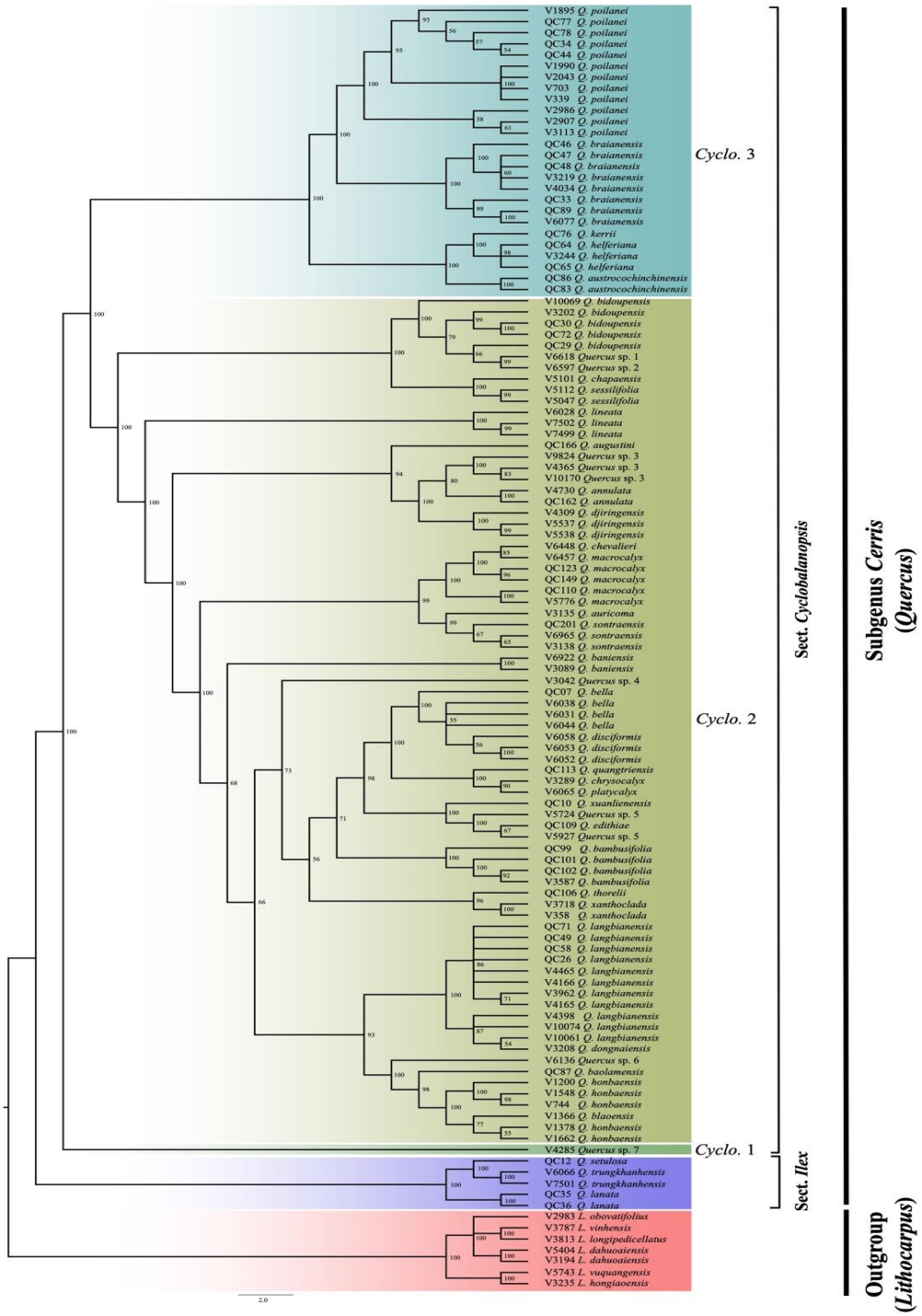

Figure 1. Phylogenetic tree of *Quercus* species in Vietnam based on data from MIG-seq

The group *Cyclo*. 3 comprised 25 accessions of the section *Cyclobalanopsis* and was supported by the absolute bootstrap value (100%). Based on morphological observation and its monophyly was confirmed by the high bootstrap value we found that this group included six species. *Quercus autrocochinchinensis* (QC86, QC83) is sister to *Q. helferiana* (QC65, V3244, QC64) and *Q. kerri* (QC76) in a clade that was supported by a 100% bootstrap value. The monophyly of *Q. braianensis* was supported and confirmed by the highest bootstrap value (100%). A total of 12 accessions were clustered in a monophyly clade that is supported by 100% bootstrap value, those are morphologically identical and were identified as *Q. poilanei.*

Morphologically, *Quercus* species in Vietnam belong to subgenus *Cerris*, consistent with the molecular research results of Hubert et al. (2014) and Yang et al. (2021). The results of the phylogenetic relationship analysis in this study also indicate that *Quercus* species in Vietnam belong to subgenus *Cerris*, encompassing two sections: section *Ilex* and section *Cyclobalanopsis*. These findings align entirely with the phylogenetic studies of Hubert et al. (2014) and Yang et al. (2021), as they posit that *Quercus* species in Asia fall under subgenus *Cerris*, comprising two sections: section *Ilex* and section *Cyclobalanopsis*.

In this study, ten samples do not resemble any previously known species of the section *Cyclobalanopsis* as well as the genus *Quercus*, but there is not enough evidence to confirm their identification. In this study, based on morphological observation we stated those samples as *Quercus* sp.1 (V6618), *Quercus* sp.2 (V6597), *Quercus* sp.3 (V9284, V4365, V10170), *Quercus* sp.4 (V3042), *Quercus* sp.5 (V5724, V5927), *Quercus* sp.6 (V6136), and *Quercus* sp. 7 (V4285) those are nested in the section *Cyclobalanopsis.*

CONCLUSION

The phylogenetic tree based on SNP data from Multiplexed ISSR Genotyping by Sequencing (MIG-seq) supports the reconstruction of a robust topology of the phylogenetic relationship between *Quercus* species in Vietnam.

Based on the results of the phylogenetic relationship analysis of this study we conclude that there is only one subgenus *Cerris* of the genus *Quercus* in Vietnam and it comprises two sections (Sect. *Ilex* and Sect. *Cyclobalanopsis*).

The results from this study reveal that section *Ilex* in Vietnam comprises three species *Q. lanata*, *Q. trungkhanhensis*, and *Q. setulosa* while the section *Cyclobalanopsis* includes at least 41 spp.

The findings of this investigation are consistent with prior research, affirming that *Quercus* species in Asia belong to the subgenus *Cerris*, which comprises two sections: section *Ilex* and section *Cyclobalanopsis*.

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Appendix. Sequences of MIG-seq primer set-1 for the 1st PCR. Underlined and **boldface** nucleotides denote tail and anchor sequences, respectively. The difference between the forward and reverse primer sets lies only in their tail sequences. SSR; simple sequence repeat

Name	Sequences (5′–3′)
Forward primers: (Tail + anchor: CTG) + SSR + anchor	
(ACT)4TG-f	CGCTCTTCCGATCTCTGACTACTACTACTTG
(CTA)4TG-f	CGCTCTTCCGATCTCTGCTACTACTACTATG
(TTG)4AC-f	CGCTCTTCCGATCTCTGTTGTTGTTGTTGAC
(GTT)4CC-f	CGCTCTTCCGATCTCTGGTTGTTGTTGTTCC
(GTT)4TC-f	CGCTCTTCCGATCTCTGGTTGTTGTTGTTTC
(GTG)4AC-f	CGCTCTTCCGATCTCTGGTGGTGGTGGTGAC
(GT)6TC-f	CGCTCTTCCGATCTCTGGTGTGTGTGTGTTC
(TG)6AC-f	CGCTCTTCCGATCTCTGTGTGTGTGTGTGAC
Reverse primers: (Tail + anchor: GAC) + SSR + anchor	
(ACT)4TG-r	TGCTCTTCCGATCTGACACTACTACTACTTG
(CTA)4TG-r	TGCTCTTCCGATCTGACCTACTACTACTATG
(TTG)4AC-r	TGCTCTTCCGATCTGACTTGTTGTTGTTGAC
(GTT)4CC-r	TGCTCTTCCGATCTGACGTTGTTGTTGTTCC
(GTT)4TC-r	TGCTCTTCCGATCTGACGTTGTTGTTGTTTC
(GTG)4AC-r	TGCTCTTCCGATCTGACGTGGTGGTGGTGAC
(GT)6TC-r	TGCTCTTCCGATCTGACGTGTGTGTGTGTTC
(TG)6AC-r	TGCTCTTCCGATCTGACTGTGTGTGTGTGAC