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ISSR and SCAR DNA markers differentiating red maple
(*Acer rubrum*) and silver maple (*A. saccharinum*)**

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Development and characterization of species-diagnostic ISSR and SCAR DNA markers
differentiating red maple (*Acer rubrum*) and silver maple (*A. saccharinum*)

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Abstract

Red maple (*Acer rubrum*) and silver maple (*A. saccharinum*) are sister species that readily hybridize in nature. No genetic or barcoding markers have been tested in these species. The main objective of the present study is to develop and characterize molecular markers distinguishing *A. rubrum* and *A. saccharinum* and to validate the hybridity of *A. freemanii* derived from their crossings using the ISSR marker system. Thirteen *A. rubrum* and seven *A. saccharinum* populations were used. Four ISSR primers including ISSR 5, ISSR 8, ISSR 10, and ISSR UBC 825 were selected to amplify genomic DNA from the two species and their hybrids. Each primer generated at least one species-diagnostic ISSR markers for a total of six. Analysis of *A. fremanii* collected from North Dakota (USA) confirmed that the genotypes screened were true hybrids between *A. rubrum* and *A. saccharinum*. These markers were cloned and sequenced. Successful sequences were converted to SCAR markers using specifically designed primers. Overall, the developed diagnostic and specific ISSR and SCAR markers are useful in the certification of these two maple species and their hybrids. They can be used in tracking the introgression of *A. rubrum* and *A. saccharinum* DNA in other hybrid trees/populations.

Key words: *Acer rubrum*; *Acer saccharinum*; *Acer freemanii*; ISSR; SCAR markers: Species-diagnostic DNA markers.

Introduction

Acer rubrum, *A. saccharinum*, and *A. freemanii*

The most well-known species of maples, include, silver maple (*Acer saccharinum*) and red maple (*Acer rubrum*). These two species intercross readily in nature producing a hybrid species, Freemans maple (*Acer freemanii*)

A. rubrum can be both a late and early successional species in many forests, where it dominates the understory and mid-canopy of many oak, and pine, in northern hardwood forests (Abrams 1998). There is high genetic diversity in this species for physiological and growth characteristics, including, cold hardiness, time of seed germination, time of budset, growth rate, stem form, fall color, as well as, drought tolerance and leaf morphology. Hence, it can adapt and survive better than other species under less water, nutrients, and light conditions (Abrams 1998). Their high genetic diversity also contributes to the widespread ecological success of the species. It reaches sexual maturity at a young age, and produces a large number of seeds with little germination requirements that are readily dispersed at maturity (Abrams 1998).

A. saccharinum is very similar to *A. rubrum* except that its leaves turn pale yellow or brown, not red, in the fall. These trees are normally found in moist areas, often along streams. They are native to eastern and central North America, in the United States and Canada (Burns and Honkala 1990). Their seedlings can survive long periods of flooding, allowing them to outcompete other species for growing space. *A. saccharinum* are often used as ornamental trees due to their early rapid growth and appealing appearance (Burns and Honkala 1990).

A. rubrum and *A. saccharinum* bloom at the same time of the season, thus providing natural hybridization between the two species. The hybrids, known as *A. freemanii*, are a good

intermediate of the parents in respect to leaf characteristics. Its growth is much more rapid than that of the *A. rubrum* seedlings, but slower than that of *A. saccharinum* (Burns and Honkala 1990).

The distinction among the three species *A. rubrum*, *A. saccharinum*, and *S. freemanii* based on morphological characteristics can be difficult especially at the seedling stage. This is in part because environmental factors also play a key role in phenotypes of plants growing in different ecological conditions.

Molecular markers are more reliable in identification of species. Phylogenetic relationships among *Acer* species confirmed that *A. saccharinum* and *A. rubrum* are sister species based on cpDNA sequences (Li et al. 2006). But the number of phylogenetic studies including these species is limited and information on their sequence is lacking.

There are several molecular markers that can be used for species identification. No genetic or barcoding markers have been tested in these species. Inter simple sequence repeats (ISSR) is a cost effective system that have been used in previous investigations. Emerging in 1994, ISSRs are DNA fragments in which the sequence is between two adjacent, identical microsatellites that are in opposite orientation (Sarwat 2012). They were first used by Zietkiewicz et al. (1994) on cultivated plant species. This marker system provides a great and reliable technique especially when studying natural populations. They are fast, affordable, economical, and, they are also easy to use (Muchugi et al. 2008). Another advantage to using the reliable ISSR primers is that no prior knowledge of the genome is required. The main disadvantage of ISSR is that it is dominant in nature and therefore, has less specificity and may lead to lower reproducibility due to the poor quality of genomic DNA; however, it is known that ISSR is still highly reproducible (Sarwat 2012). For example, they have been successfully used

in detection of somaclonal variation, genetic stability (Lata et al. 2010), gene tagging (Ammiraju et al. 2001), identification of cultivar (Nagaraju et al. 2002), identification of hybrids (Lin et al. 2010), phylogenetic studies (Paris et al. 2003), genetic relatedness (Rajwade et al. 2010), as well as, in assessing production quality (Wu et al. 2010). It has been reported that ISSRs are more polymorphic and informative compared to restriction fragment length polymorphisms (RFLP) and amplified fragment length polymorphism (AFLP) (Sarwat 2012). Sequence characterized amplified regions (SCAR) markers developed from ISSR and other molecular markers are very sensitive and highly reliable (Nkongolo et al. 2005). They can aid in solving any ambiguities in identifying targeted species.

The main objective of the present study is to develop and characterize ISSR and SCAR markers distinguishing *A. rubrum* and *A. saccharinum* and to validate the hybridity of *A. freemanii*.

Materials and Methods

Plant sampling and processing

Certified seeds of *Acer rubrum* from different sources were provided by the National Tree Seed Centre (Natural Resources Canada, Fredericton). They include seedlots 200010442, 20011024, 20011031, 20021067, 20081002, and 20011037. For *A. saccharinum*, certified seeds (lot 1823857) originating from Kentucky were provided by Shieffield seed Co. Inc (New York). Additional *A. rubrum* and *A. saccharinum* samples were collected as leaves from different sites in the Northern Ontario in ecological areas specific for each species. For *A. rubrum*, the sampling sites include Kelly Lake, Azilda, Capreol, Kingsway, St. Charles, Wahnapiatae Sudbury Hydro, and Falconbridge. For *A. sacharinum*, leaves were collected from trees growing on four sites

including Laurentian University campus, Laurentian conservation area, Madison Avenue, and Westmount (New Sudbury). On each site, 10 trees were selected. About 10 to 20 leaves from each tree were collected. Seeds of *A. rubrum* and *A. saccharinum* from different provenances in Ontario, New Brunswick, and Quebec were also used for this marker development and validation (Table 1). Seeds of *A. freemanii*, collected in North Dakota from controlled crossings (Lot number 1821454), were provided by Sheffield Seed Company, New York, USA. All the seeds were germinated in “Petawawa” germination boxes and seedlings were grown in a deep tray with soil. The leaves were wrapped with aluminum paper, then submerged into liquid nitrogen, and stored at approximately -20°C until DNA extraction.

DNA Extraction

Genomic DNA was extracted following the protocol described by Nkongolo et al. (2005). One gram of the frozen plant material was grinded into a fine powder in liquid nitrogen, using a mortar and a pestle. Each sample was suspended in 15 mL of CTAB buffer (pH 8.0 with 1% PVP and 0.02% β -mercaptoethanol) and incubated in a water bath at 60°C for 45 minutes. Once an emulsion was attained, an equal amount of chloroform: octanol mix (24:1) was added to each tube, balanced, and then vigorously inverted for 3 minutes. The samples were then centrifuged at 12,500 rpm for 15 minutes at room temperature (25°C). The aqueous layer of each sample was then transferred to their respectively labelled tubes. An equal amount of chloroform:octanol mix was again added to each tube, and once emulsion was attained, the samples were centrifuged at the same speed and temperature as previously mentioned for 5 minutes. The aqueous layer was transferred to new tubes, with equal volume of chloroform: octanol mix added, then, inverted, balanced, and centrifuged at 12, 500 rpm for 5 minutes at room temperature. The supernatant was transferred to fresh tubes, where 10 mLs of isopropanol had been added. The tubes were

gently inverted and balanced, then placed in the centrifuge at 6,500 rpm for 25 minutes at 4°C. The tubes were then drained with a Pasteur pipette, and 5 mL of 70% ethanol was added, and the solution was incubated at room temperature for 5 minutes. The tubes were then drained for a second time, and the bubbles were carefully vacuumed up. Once all the ethanol was evaporated, the DNA pellet that had formed was suspended in about 200-500 µL (depending on the size of the pellet) of 1xTE (Tris-EDTA) buffer. The tubes with the resuspended pellet of DNA were stored at 4°C until the following day. For *A. freemanii*, genomic DNA was extracted from embryos because of poor seed germination following the QIAGEN DNeasy protocol

Amplification of ISSR markers

Initially, fifteen ISSR primers were tested for preliminary amplification of *A. rubrum*, *A. saccharinum*, and *A. freemanii*. PCR analysis was carried out following the procedure described by Nkongolo et al. (2005), Theriault et al. (2014), and Moarefi et al. (2018). Each PCR reaction was performed using a total of 25 µL volumes containing a master mix of 11.4 µL distilled water, 2.5 µL MgSO₄, 2.1 µL 10x buffer 0.5 µL of dNTPs (equal parts dTTP, dATP, dCTP, dGTP), 0.5 µL of ISSR primer, a *Taq* mix of 3.475 µL distilled water, 0.4 µL 10x buffer, and 0.125 µL *Taq* polymerase (Applied Biosystems) and 4 µL standardized DNA. For each primer, a negative control reaction was included where ddH₂O was added instead of DNA. All samples were covered with one drop of mineral oil to prevent evaporation and amplified with the Eppendorf Mastercycler gradient. The program was set to a hot start of 5 minutes at 95 °C followed by 2 minutes at 85°C after which the *Taq* mix was added. This was followed by 42 cycles of 1.5 minutes at 95 °C, 2 minutes at 55 °C and one minute of 72 °C, and a final extension of 7 minutes at 72 °C. Samples were then removed from the thermocycler and placed in the -20 °C freezer until further analysis. All PCR products were loaded into 2% agarose gel in 0.5X Tris-Borate-

EDTA (TBE) buffer. After the DNA samples were amplified, they were separated for analysis on a 2% agarose gel in 0.5x TBE with ethidium bromide and run at 3.14V/cm.

Five μ L of 1x loading buffer were added to the PCR products and 10 μ L of this solution were loaded into the wells of the gel. The gel was run as described above, documented with the Bio-Rad ChemiDoc XRS system and analyzed with Image Lab Software. Only the ISSR primers which gave consistent profiles across the populations and also those that appeared to have diagnostic markers were selected for further analysis.

Cloning and sequencing

Species-diagnostic ISSR bands were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen) and quantified using the Saran wrap method (Sambrook et al. 1989; Vaillancourt et al. 2008). They were cloned into the pGEM-T® Easy Vector System II (Promega, Madison, WI). *Escherichia coli* JM 109 competent cells were transformed with the recombinant vectors and were then plated onto LB/ampicillin/IPTG/X-Gal plates. Positive colonies were determined by blue/white screening. Plasmids from randomly selected white colonies were extracted using the lysis by alkali protocol from Sambrook et al. (1989) with few modifications. The vectors containing the fragments of the correct size were sequenced at University of British Columbia Sequencing and Bioinformatics Consortium. The sequenced data were analyzed using ABI Prism sequencing analysis version 3.3 (PE Biosystems). For each sequence, a pair of target primers flanking the insert region was designed and synthesized by Life Technologies Inc.

Results

Sequences of the 15 primers tested along with their GC content are described in Table 2. Four of these primers screened generated consistent banding profiles. They include ISSR 5, ISSR 8, ISSR 10, and ISSR UBC 825. A band that was present in all *A. rubrum* samples and absent in all the *A. saccharinum* was considered a diagnostic marker for *A. rubrum*. Likewise, an *A. saccharinum* diagnostic marker is present in all the samples from this species and absent in *A. rubrum* samples. For ISSR 5, a diagnostic DNA marker was found for *A. saccharinum* at 950 bp, and at 490 bp for *A. rubrum*. For ISSR 8, a diagnostic marker of 2,000 bp was identified in *A. saccharinum*. For ISSR 10, one *A. saccharinum* DNA marker of 1,400 bp and one *A. rubrum* DNA band of 1,900 bp were detected (Table 3). Finally, ISSR 825 generated a 500 bp band diagnostic for *A. rubrum*. Each species-diagnostic marker was validated three times. Some species-diagnostic ISSR markers are illustrated in Figures 1, 2, 1S, and 2S.

The same four primers, ISSR 5, ISSR 8, ISSR 10, and ISSR UBC 825, were used to confirm the hybridity of 40 genotypes from an *A. freemanii* population. Both the *A. rubrum* and *A. saccharinum* DNA diagnostic markers were present in *A. rubrum* x *A. saccharinum* (*A. freemanii*) genotypes screened. PCR amplifications for *A. freemanii* are depicted in Figures 2 and 2S.

To assess the species-specificity of ISSR markers, pairs of primers flanking cloned ISSR fragments were designed and used to target these sequences in genomic DNA of *A. rubrum* and *A. saccharinum* in more sensitive PCR assays. The main characteristics of designed primers are described in Table 4. Amplification of *A. saccharinum* DNA with the Ac-Sac-UBC-825 primer pair generated a strong species-specific SCAR since it was not identified in any *A. rubrum* that we screened (Fig. 3). The consensus sequence of this marker has been registered in the National

Center for Biotechnology Information (NCBI) Genbank in the Bethesda (Maryland, USA) under the accession number MK287621 (Fig. 4). BLAST search results reveal no matching sequence for this marker in any database. Amplification of *A. rubrum* DNA with the Ac-R-UBC-825 primer pair generated faint bands.

Discussion

The reliability of the ISSR marker system has been confirmed in different investigations. Prevost and Wilkinson (1999) reported that ISSR can generate more polymorphisms than RFLP and RAPD, thus providing reliable and promising information. ISSR revealed clear, distinct, and sufficient information needed to characterize tree populations in a study reported by Nudin et al. (2017). Balasaravanan et al. (2006) successfully used ISSR marker system to distinguish six *eucalyptus* species of seaweed, as many of their characteristics show similarities. Salis et al. (2017) demonstrated the usefulness of ISSR molecular marker technique in distinguishing between species of citrus rootstocks.

Species-specific wheat (*Triticum aestivum*) and rye (*Secale cereale*) ISSR markers have been described by Vaillancourt et al. (2008). Nkongolo et al. (2005, 2012) used ISSR and RAPD marker systems to certify the identity of spruce (*Picea spp.*) trees. They were able to develop species-diagnostic markers, and validate that interior spruce trees analyzed were true *P. glauca* and *P. engelmannii* hybrids. Interestingly, they demonstrated that one of the declared *P. glauca* x *P. mariana* hybrids in many studies based on morphological characteristics was not a hybrid rather a true *P. glauca*.

In the present study, ISSR primers used generated a high level of polymorphic loci. The data were highly reproducible and provided strong support for the use of ISSR for hardwood

species differentiation. Although the comparative analysis of these two species (*A. rubrum* and *A. sacharum*) is lacking in the literature, one study demonstrated using haplotypes that *A. rubrum* and *A. saccharinum* had a distinct phylogeographic group (Saeki et al. 2011). Krahling et al. (1993), demonstrated using RAPD that *A. rubrum* has unique sequences that differentiate it from other maple species.

Skepnier and Krane (1998) compared black maple (*A. nigrum*) and sugar maple (*A. sacharum*) using RAPD system. They showed that although classified as different species, *A. nigrum* and *A. saccharum*, have similar RAPD marker profiles. These results could be because the primers used might not target differential regions of the genomes rather than the lack of genetic differences between the targeted species.

Although all the species–diagnostic markers described in the present study are useful, the development of a SCAR marker provided a more practical tool for screening a large number of samples. Two species-diagnostic ISSR markers were successfully converted to a species-specific SCAR marker, but only one sequence was deposited in the NCBI genebank as it was very clean. There exists a couple of reasons that could explain why diagnostic markers such as some of those developed in the present study once converted to a SCAR marker are not species-specific. The appearance of a band in one species and its absence in another could result from competitions among DNA fragments during amplification (Nkongolo et al. 2003; Lorenz 2012). In fact, amplified products which are complementary to each other are stabilized by internal base pairing that could prevent amplification by out-competing the binding of random primers (Nkongolo et al. 2003; Lorenz 2012); this is the most serious problem that leads to the incorrect interpretation of results. Formation of secondary structures including hair pin, by DNA fragments could also have a critical impact on the amplification. Such structures, if formed,

might hinder the amplification of potential target regions by making the primer binding site inaccessible to primer and /or the polymerase. However, the frequency of heteroduplex formation of bands with homologous sequences is very low. Since the SCAR system is a more sensitive technique, the above problems will not interfere with the amplification of sequences even if they are present in a low copy number.

The markers developed in the present study will certainly be a reliable tool for ecological studies. Saeki et al. (2011) reported based on cpDNA analysis that *Acer rubrum* showed greater haplotype diversity than *A. saccharinum* because of its greater geographic range, and ecological, and morphological variation. Their study provides evidence of introgression where these two species are sympatric, which led to an increase in haplotype diversity in both species. The diagnostics ISSR markers and species-species SCAR markers developed in this study would be very useful in validating the hybridity of populations and trees in sympatric congeneric species. They can be also suitable for screening *Acer* seedlings in nurseries in a revegetation program to make sure that the right genotypes are planted in the appropriate ecological niches.

Moreover, the identification of hybrids between *A. rubrum* and *A. saccharinum* (Freeman maples) has been very difficult. Because of a high level of morphological variation within both parent species, and the lack of knowledge of the particular parent trees involved in the cross, only trained botanists and horticulturists, are able to classify putative hybrids in some stands (Santamour 1993). The development of these species diagnostic markers is also relevant because of the new wave of interest in freeman maple as potential superior trees for urban planting.

The high variability observed in both species can be in part ascribed to the existing cytological variations. Although recent cytological studies are lacking, it is well established that *A. saccharinum* is tetraploid ($2n = 4x = 42$) while *A. rubrum* is hexaploid ($2n = 6x = 78$) and octoploid ($2n = 8x = 104$) (Santamour 1965). Aneuploid genotypes have been reported in some stands for both species (Santamour 1965). The chromosome number in Freeman maples is highly variable because of significant differences between the two parental species for chromosome number. The molecular markers developed in this study will certainly be the most cost effective approach in certifying Freeman trees in mixt stand. But Genomic fluorescent *in situ* hybridization (GISH) will be the best approach to determining chromosome composition for Freeman tree maples (Nkongolo et al. 2009).

Conclusion

In the present species, four ISSR markers generated strong and reproducible ISSR markers in *A. rubrum*, *A. saccharinum*, and *A. freemanii*. The species-diagnostic and specific ISSR markers developed were successfully used to certify the validity of the *A. rubrum* x *A. saccharinum* hybrids commonly known as *A. freemanii*. These markers were converted to SCAR markers using specifically designed primers flanking the marker sequences. SCAR markers derived from *A. saccharinum* and *A. rubrum* were developed and characterized and can be a very reliable tool in ascertaining or tracking the presence of these maple DNA in a tree or population.

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Conflict of interest

Authors declare that they have no conflict of interest.

Compliance with ethical standards

This article does not contain any studies with human subjects or animals performed by any of the authors.

Draft

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Figure caption

Figure 1. ISSR amplification pattern of *Acer rubrum* and *Acer saccharinum* DNA with primer ISSR 10. Lanes 1 and 40 contained 1-kb plus DNA ladder; lanes 2-21, *Acer saccharinum*; Lanes 22-38, *Acer rubrum*. The arrows indicate species –diagnostic ISSR markers for *A. rubrum* and *A. saccharinum*.

Figure 2. ISSR amplification pattern of *Acer freemanii* DNA with primer ISSR 8. Lane 1 contained 1-kb plus DNA ladder; lanes 2 and 3, *Acer saccharinum* and *Acer rubrum* respectively. Lanes 4 -9, *Acer freemanii*. The arrow shows the presence of *A. saccharinum* diagnostic marker in *A. freemannii*.

Figure 3. PCR amplification of *Acer saccharinum* and *Acer rubrum* with Ac-Sach UBS 825 (forward and reverse) primers. Lane 1 contained 1-kb plus DNA ladder, lanes 2-11 contain *Acer saccharinum* and 12-21 contain *Acer rubrum* respectively, lane 22 is a no template control. The arrow indicates the SCAR marker in *A. saccharinum*. Note the absence of bands in *Acer rubrum* (Lanes 12 to 22).

Figure 4. Consensus sequence of an Ac- Sac -UBC 825 ISSR marker (680 bp) specific for *Acer saccharinum*.

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Figure 1S. ISSR amplification patterns of *Acer rubrum* and *Acer saccharinum* DNA with primer ISSR 825. Lane 1 contained 1-kb plus DNA ladder; lanes 2-21, *Acer saccharinum*; Lanes 22-40, *Acer rubrum*. The arrows indicate species-diagnostic markers for *A. saccharinum*.

Figure 2S. ISSR amplification pattern of *Acer freemanii* DNA with primer ISSR 10. Lane 1 contained 1-kb plus DNA ladder, lanes 2 and 3, *Acer saccharinum* and *Acer rubrum* respectively. Lanes 4-9, *Acer freemanii*. The arrows indicate the presence of *A. rubrum* diagnostic marker in *A. freemanii*.

Table 1. List of provenances of tree species tested for the presence of the UBC 825 *Acer saccharinum* and *A. rubrum* diagnostic/ specific ISSR and SCAR markers.

Species	Provenance	<i>A. saccharinum</i> diagnostic /specific UBC 825 ISSR and SCAR marker	<i>A. rubrum</i> diagnostic/specific UBC 825 ISSR and SCAR marker
<i>Acer rubrum</i>	Spencer Settlement, NB, CAN	Absent	Present
	Acadia Research Forest , NB, CAN	Absent	Present
	Larry Brook, NB, CAN	Absent	Present
	Rooth, NB, CAN	Absent	Present
	Oxford, NS, CAN	Absent	Present
	Cold brook, NB	Absent	Present
	Daisy Lake, Sudbury, ON, CAN	Absent	Present
	Laurentian, Sudbury, ON, CAN	Absent	Present
	Wahnapiatae, ON, CAN	Absent	Present
	Kukagami, ON, CAN	Absent	Present
	Falconbridge, ON, CAN	Absent	Present
	St. Charles, ON, CAN	Absent	Present
	Onaping Falls, ON, CAN	Absent	Present
	Capreol, ON, CAN	Absent	Present
<i>Acer saccharinum</i>	Laurentian Conservation, ON, CAN	Present	Absent
	Madison Av-Sudbury, ON, CAN	Present	Absent
	Westmount, Av. –Sudbury, ON, CAN	Present	Absent
	Nairn Centre, ON, CAN	Present	Absent
	Backus Wood, ON, CAN	Present	Absent
	Montreal Botanical Garden, QC, CAN	Present	Absent
	La Vallée-du-Richelieu, Otterburn Park Montréal, QC, CAN	Present	Absent
	Kentucky, USA	Present	Absent

CAN = Canada; NB = New Brunswick; ON = Ontario; QC = Quebec.

Table 2. Sequence of ISSR primers used to amplify *Acer rubrum* and *Acer saccharinum* DNA

Primer	Sequence (5' – 3')	G/C content (%)	Annealing temperature (°C)
ISSR 1	(AG) ₈ RG	53	55
ISSR 2	(TC) ₈ YC	50	55
ISSR 3	(GAC) ₄ G	69	55
ISSR 4	(CGT) ₄ C	69	55
ISSR 5	(ACG) ₄ AC	64	55
ISSR 6	(TTG) ₅ CB	39	55
ISSR 7	(AGG) ₅ GY	65	55
ISSR 8	(AGAT) ₅ GY	27	55
ISSR 9	(GATC) ₃ GC	57	55
ISSR 10	(CTT) ₅ (CCT) ₆ CT	51	55
ISSR HB 12	(CAC) ₃ GC	73	55
UBC 823	(TC) ₈ C	52	55
UBC 825	(AC) ₈ T	47	55
835	(AG) ₈ YC	50	55
844	(CT) ₈ RC	53	55

Table 3. Species-diagnostic markers identified in *Acer rubrum* and *A. saccharinum*

Primers	Species-diagnostic marker size (bp)	
	<i>Acer rubrum</i>	<i>Acer saccharinum</i>
ISSR 5	450	950
ISSR 8	-	2000
ISSR 10	1900	1400
ISSR UBC 825	500	-

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Table 4. Sequences and characterization of designed pairs of primers *A. saccharinum* and *A. rubrum* diagnostic markers

Primer Name	Primer Sequence (5'-3')	Tm	Targeted Fragment (bp)	Species Specificity
Ac-Sac-UBC825 F	TTATGGAAACCTGGGAGCTG	54°C	489	<i>Acer saccharinum</i>
Ac-Sac-UBC825 R	CATCGAATCTTCGCCATTGT	54°C		
Ac-R-UBC825 F	TTCATTGCTTTGTGCCTCTATG	54°C	215	<i>Acer rubrum</i>
Ac-R-UBC825R	AGCCAGAAGAACAGCCCAGT	58°C		

AC-Sac-UBC 825 and Ac-R-UBC 825 represent designed primers targeting *A. sacharinum* and *A. rubrum* diagnostic marker, respectively to generate SCAR markers.

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170x103mm (300 x 300 DPI)

199x199mm (300 x 300 DPI)

150x103mm (300 x 300 DPI)

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1      1  CACACACACA CACACTTTAT GGAAACCTGG GAGCTGAAAA TCATGAGAAC TTGATAAAGT
2      61  GCGGGTGCCA ACCGCACTCC ACTCGCACTC ATGCCGAAAC CCGGGCGCTG TGCAGATGAA
3     121  GAATCAATAA AGTGCAGGTC TCAACCGTAC TTACCCGTGC TTTAAGGTGG AAATTCCTCA
4     181  GTGCGTTGGT AAGCACACCC GCGCACTACC AGCGCATTTT GTGCCAGATT TCGCCATGTC
5     241  AGCATTTTGT CTGACCTAAT TTCGAGAATA TTATAAATAT TTGTTACCGA AAAATCGTCA
6     301  CGAGGGGTGA AAATTCCGAT TAACAACCTA AAAGGGACCA AGGTCGAAAT TGACCAAAAT
7     361  CGGAGAGGTC TCACATGGTT TGAGACCTAT GCATGCGAAG ACATAATACG AAGAACTCTG
8     421  AACATAAGAT ACATGCAACA AAGAGGCAAA ATACTGTTTT ATTCATACAA TATTTTTTCT
9     581  ACGTTACAAT GCGGAAGATT CGATGCATTA TCAGAGGATA TCAATGGAAA ACTATCCTAT
10    641  GGGACTGATG ATCCTCATTC TGCTCTCGAG TGTGTGTGTG TGTGT

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Figure 4. Consensus sequence of an Ac- Sac -UBC 825 SCAR marker (680 bp) specific for *Acer saccharinum*.

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