Sex-linked molecular markers for two cold-hardy kiwifruit species, *Actinidia arguta* and *A. kolomikta*

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Summary

Developing improved fruiting varieties of kiwiber-ry (*Actinidia arguta*) and other species of cold-hardy kiwifruit (e.g., *A. kolomikta*) is highly resource-inten-sive due to the dioecy, extended juvenile phase (3–5 years), and vegetative vigor of these woody, vining species. The ability to identify gynoeious plants at the seedling stage is therefore desired as a means of increasing the resource-use efficiency of breeding programs. In this study, sex-linked polymorphisms in both species were identified through reference-inde-pendent genotyping-by-sequencing (GBS) analysis; validated in independent populations; and convert-ed into cost-effective, gel-based PCR markers appro-priate for marker-assisted breeding programs. The marker for *A. arguta*, designed using male-allele-spe-cific primers based on a SNP within a 157 bp GBS amp-licon, exhibits perfect sex-linkage across a compre-hensive set of kiwiber-ry germplasm currently avail-able through the US nursery trade and the USDA's National Plant Germplasm System (NPGS). By multi-plexing conserved primers for a co-located internal 81 bp control amplicon, this male-specific marker was made effectively co-dominant, thereby enhanc-ing scoring accuracy. Similarly, the 161 bp marker for *A. kolomikta* was designed with male-allele-specific primers based on a 3 bp indel within a 270 bp GBS fragment and exhibits perfect sex-linkage throughout the available *A. kolomikta* germplasm in North Ameri-ca. Both markers were validated in independent pop-ulations and are now being used for high-throughput sex-screening assays of seedling populations.

Keywords

kiwiber-ry, cold-hardy kiwifruit, *Actinidia arguta*, *Actinidia kolomikta*, marker-assisted selection, sex screening

Introduction

Although the genus *Actinidia* is comprised of an esti-mated 76 species (Ferguson and Huang, 2007) native to a wide range of habitats across eastern Asia, nearly all of the >US\$ 2 billion global kiwifruit trade (FAO, 2013) is based on cultivars of two closely related and recently domesticated temperate species: *A. chinensis* var. *deliciosa* and, increasingly, *A. chinensis* var. *chinensis* (Ferguson, 2013). While cold-hard-ier members of the genus, namely *A. arguta* and *A. kolomik-ta*, have long been recognized for their economic potential, particularly at higher elevations and in more northern lati-tudes (Goodale, 1981), these species have received relatively

Significance of this study

What is already known on this subject?

- Like all kiwifruit species within the diverse *Actinidia* genus, the cold-hardy species *A. arguta* and *A. kolomikta* are dioecious. Given the long juvenile periods of these woody, perennial lianas, dioecy presents a significant and fundamental inefficiency to breeding programs, one that could be solved through the use of sex-linked molecular markers to identify male and female plants at the seedling stage.

What are the new findings?

- Using diverse association mapping panels and independent validation populations, this study develops, validates, and reports the full details of a low-cost, sex-linked molecular marker for each species. By providing the first publically-available molecular markers specifically developed for *A. arguta* and *A. kolomikta*, this study paves the way for the marker-assisted improvement of these species.

What is the expected impact on horticulture?

- By adopting the use of these markers, marker-assisted breeding programs for these emerging horticultural crop species can greatly improve their resource efficiency, thereby accelerating the development of improved cultivars.

little attention by formal breeding programs in the United States since their initial introduction into the country as or-namentals in the 1870's (Ferguson and Seal, 2008). On one hand, this lack of investment is surprising, given the growing number of examples of the commercial viability of extant selections and the numerous desirable traits of their small, grape-sized fruits, including impressive nutritional profiles (Nishiyama et al., 2004; Crowhurst et al., 2008; Huang et al., 2013), exceptional flavor (Ferguson, 2013), and hairless, ed-ible skins. On the other hand, systematic plant breeding pro-grams are long-term, high-investment propositions under the best of conditions; and the risks are inherently higher for a novel, resource-intensive crop with essentially no current commercial base. Not only do these vigorous, woody lianas require substantial physical infrastructure (e.g., trellising), intensive vine management (i.e., training and multiple prun-ings per year), and significant physical space (> 14 m² per plant under commercial production systems); they also re-quire up to five years or more before reaching reproductive maturity.

Complicating matters further is the fact that, although most angiosperms are hermaphroditic (Friis et al., 1987;

Renner and Ricklefs, 1995), all *Actinidia* species are reported to be functionally dioecious (Seal and McNeillage, 1988). This trait presents a fundamental inefficiency to breeding programs, particularly in light of the ~60–70% male bias routinely observed in randomly segregating populations (R. Guthrie and D. Jackson, pers. commun.). With neither sexually dimorphic traits at the seedling stage nor cytologically-evident sex chromosomes within *Actinidia* spp. (Ming et al., 2011), the sex of any given kiwifruit vine is discernible only once it reaches reproductive maturity. Sex-linked molecular markers developed in other dioecious genera [e.g., *Asparagus* (Reamon-Buettner and Jung, 2000), *Carica* (Deputy et al., 2002), and *Cannabis* (Mandolino et al., 1999)] demonstrate the potential of enhancing breeding efficiency by allowing an effective increase in population sizes, given resource constraints. Similarly in *A. chinensis* var. *chinensis*, a sub-telomeric sex-associated locus was discovered with tightly linked male (SMY) and female (SMX) associated loci (Fraser et al., 2009), though the causative genes have yet to be identified and the markers unfortunately fail to exhibit sex-linkage in either cold-hardy species, *A. arguta* or *A. kolomikta*.

While the specific mechanisms underlying sex determination in *Actinidia* spp. remain unknown, it has long been observed that male plants within the genus possess staminate flowers with rudimentary ovaries while female plants appear hermaphroditic but are male-sterile (Ferguson, 2013). Due to the apparent disomic segregation of ovary abortion (O) and pollen fertility (P) across various ploidy levels (Testolin et al., 1999), these two traits have been hypothesized to be controlled by two tightly-linked genes, with *op/OP* individuals developing as male and *op/op* individuals as female (Ainsworth, 1999). This genetic model finds support in the rare occurrences of both sterile (putative *op/Op*) and hermaphroditic (putative *op/op*) individuals (Ferguson, 2013), as well as in the observed linkage between the sex-associated SMX and SMY regions identified in *A. chinensis* var. *chinensis* (Fraser et al., 2009).

As no marker-assisted selection protocols are currently available to support public breeding efforts in the relatively understudied species *A. arguta* and *A. kolomikta*, this investigation was undertaken to develop cost-effective, PCR-based assays for sex-linked loci in both species. In light of the recent ban on importing additional *Actinidia* germplasm into the US due to concerns over *Pseudomonas syringae* pv. *actinidiae* (USDA, 2010), the target germplasm for this study consists of all available accessions in the US nursery trade and the USDA's National Plant Germplasm System (NPGS) (see Melo et al., 2017).

Materials and methods

Plant material

For the initial genotyping-by-sequencing (GBS) association analysis, leaf tissue was sampled from the complete set of non-redundant accessions of the North American collection of cold-hardy kiwifruit germplasm, as described by Melo et al. (2017). Specifically, this set included 60 accessions of *A. arguta* (42 females, 17 males, 1 unknown) and 13 accessions of *A. kolomikta* (10 females, 3 males) (see Supplemental information – Table S1). For subsequent marker validation, the following three independent populations were used: 1) An open-pollinated population of 17 mature, half-sib *A. arguta* lines (10 females and 7 males; mother: *A. arguta* cv. Ogden Point) from the University of Minnesota

Landscape Arboretum Horticultural Research Center (HRC); 2) An open-pollinated population of 21 mature, half-sib *A. arguta* lines (11 females and 10 males; mother: *A. arguta* cv. Hortgem Tahi) from Kiwi Berry Organics Co. in Danville, PA; and 3) An open-pollinated population of 21 mature, half-sib *A. kolomikta* lines (10 females and 11 males; mother: *A. kolomikta* cv. Nahodka) from the HRC.

DNA isolation, library preparation, and sequencing

Genomic DNA was extracted using a modified CTAB protocol. Specifically, 100 mg of fresh, healthy leaf tissue was lyophilized and ground to a powder in a Retsch mill, using three 3-mm tungsten carbide beads per 1.1 ml tube. The resultant powder was incubated at 65°C in 750 µl of DNA extraction buffer (CTAB, 1% polyvinylpyrrolidone and 2% 2β-mercaptoethanol) for 45 minutes, with occasional mixing. After adding 750 µl of chloroform:isoamyl alcohol (24:1 v/v) and mixing by gentle inversion, tubes were centrifuged at 10,000 rpm for 10 minutes and the aqueous phase recovered. 10% volume of 65°C CTAB/NaCl solution was added, followed by a second chloroform:isoamyl alcohol step. Nucleic acids were precipitated with an equal volume of 95% isopropanol and centrifuged at 13,000 rpm for 10 minutes. The recovered pellet was washed twice with 700 µl of 70% ethanol, air dried, and resuspended in 50 µl of 10 mM Tris. Prior to GBS library preparation, the isolated gDNA was purified using Zymo DCC-10 clean-up columns (Zymo #D4011).

Multiplexed GBS libraries were prepared according to the two enzyme (PstI - MspI) protocol described by Poland et al. (2012). Using a set of 6–10 bp barcodes from that protocol, the 60 accessions from *A. arguta* and 13 accessions from *A. kolomikta* were multiplexed and sequenced on an Illumina HiSeq 2500 at the Hubbard Center for Genome Studies, University of New Hampshire. FASTQ files of the 150 bp paired-end (PE) sequence data were generated using CASAVA 1.8.3; and all parsed, high-quality, PE reads were deposited in the NCBI Sequence Read Archive. Detailed information about the 73 accessions in this study, such as genotype names, NPGS accession numbers, reported and observed gender information, the number of high quality PE reads, the number of variants called for each genotype, the NCBI SRA submission IDs, etc., can be found in Supplemental information – Table S1.

Genotyping

The CASAVA-processed, raw sequence data from both species were submitted for analysis to version 2.1 (SNP + indel functionality) of the GBS-SNP-Calling Reference Optional Pipeline (GBS-SNP-CROP; Melo et al., 2016). As discussed in more detail by Melo et al. (2016), the pipeline calls Trimmomatic (Bolger et al., 2014) to trim reads based on a sequence of three contiguous nucleotides with an average Phred score $Q \leq 30$. High-quality reads at least 32 bp in length were then demultiplexed into read pairs, by genotype. For this study, in an attempt to avoid dominant tags present only in male genotypes, the most read-abundant female genotype from each species (cv. ORUS 2-16 for *A. arguta* and cv. Matonaya for *A. kolomikta*) was chosen to build a reduced-representation reference (i.e., Mock Reference; see Melo et al., 2016) to facilitate GBS read mapping and variant (SNP and indel) discovery. After alignment to the mock reference using BWA-mem (Li and Durbin, 2009), SAMtools (Li et al., 2009) was used to retain only those read pairs that mapped in proper orientation with no supplementary or duplicate alignments for variant calling. As the two species evaluated have different ploidy levels (Melo et al., 2017), we used the respective,

recommended GBS-SNP-CROP parameters for step 7 to call variants for diploid (*A. kolomikta*) and tetraploid (*A. arguta*) species.

A simple search for SNP and indel markers exhibiting homozygosity for all female accessions and heterozygosity for >95% of the male accessions yielded sets of candidate sex-associated markers for *A. arguta* and *A. kolomikta*. The relaxed (>95%) heterozygosity requirement for male accessions was chosen due to the fact that GBS, by virtue of its non-uniform read depth across all loci, can incorrectly score heterozygotes as homozygotes (see Melo et al., 2016). For each candidate marker, the associated FASTA entry from the Mock Reference (i.e., the consensus GBS fragment or centroid) was examined to understand the sequence context of the putative male/female polymorphism and guide PCR-based primer design for confirmation within the original population and validation in independent populations.

PCR marker development and validation

Primers for polymorphic amplicons putatively associated with sex were designed using Primer3 (Rozen and Skaletsky, 2000) and initially tested on a small sample of accessions of known gender from the same population used for GBS analysis (see Supplemental information – Table S1). All PCRs were conducted in a total reaction volume of 20 µl (0.25 mM of each primer, 100 µM of each dNTP, 0.75 U Taq DNA Polymerase, 10× standard Taq buffer [NEB N0273], and 50–100 ng of template DNA). Cycling conditions consisted of 5 min at 94°C; 32 cycles of 30 s at 94°C, 30 s at the appropriate primer annealing temperature, and 15 s at 68°C; followed by a final 5-minute elongation step. Amplified products were separated at 75 V on a 2% TBE/EtBr agarose gel for 60 min and imaged with UV transillumination. Following successful amplification on the initial sample of accessions, promising markers were used to screen the entire US kiwiberry collection, as well as the independent segregating populations.

Results

Bioinformatics analysis and primer design

Using only the single most read-abundant genotype to build each species-specific Mock Reference, the GBS-SNP-CROP pipeline (v2.1) called a total of 32,222 (mean depth $D = 60.24$) and 12,486 ($D = 57.06$) variants for *A. arguta* (60 accessions) and *A. kolomikta* (13 accessions), respectively. For *A. arguta*, of the total variants called, 30,468 were SNPs and 1,754 were indels, while for *A. kolomikta* the pipeline called 12,067 SNPs and 419 indels. These sets of variants were called using 286.3 and 69.6 million PE high quality reads for *A. arguta* and *A. kolomikta*, respectively (Table 1). The two species exhibited similar values of overall loci heterozygosity (Hetero), homozygosity (Homo), and missing data (NA), with the intraspecific averages being 27.7% (Hetero), 60.4% (Homo), and 11.9% (NA) for *A. arguta* and 35.3% (Hetero), 58.0% (Homo), and 6.7% (NA) for *A. kolomikta* (Table 1).

The initial association analyses yielded three candidate sex-linked clusters in *A. arguta* and four in *A. kolomikta* [hereafter noted with the prefixes aC (*arguta* Cluster) and kC (*kolomikta* Cluster), respectively] (Tables 1 and 2), several of which contained multiple polymorphisms (for full details, see Supplemental information – File S1). Sex-linked sequences were prioritized for marker development based on practical constraints of primer design as well general considerations of efficiency, reproducibility, and ease of scoring in the lab. In general, allele-specific primers were pre-

ferred over CAPS markers, as we wished to avoid the extra cost (time and money) of restriction digestion. Additionally, length polymorphisms were preferred over SNPs due to their relative ease of scoring on gels, and the molecular weights of amplicons were prioritized with regard to downstream gel resolution. Three putative sex-linked polymorphic sequences could not be immediately developed into PCR markers due to variant locations within centroids that restricted primer design (clusters aC70626, kC693, and kC1273). The majority of putative sex-linked variants, however, were located more than 20 bp from either end of a centroid, thereby permitting the placement of primers on both sides of the variant (Table 2).

Experimental primer pairs were designed for variants within two clusters in *A. arguta* [aC36306 (1 SNP) and aC123485 (1 indel)] and two clusters in *A. kolomikta* [kC48400 (2 SNPs) and kC72369 (1 indel); see Table 2]. For variants involving no length polymorphism (e.g., SNPs), primer pairs were designed to amplify only male-specific sequences in order to avoid a second, costly restriction digestion step (i.e., CAPS marker). To accomplish such male-specific amplification, one of the primers (either forward or reverse) was designed such that the final nucleotide on the 3' end matched only the male variant and the antepenultimate nucleotide on the 3' end mismatched both male and female variants, effectively blocking extension of female variants. Two candidate variants were indels and required no such designed mis-match. In cluster aC123485, a co-dominant primer pair was designed around a 10 bp indel, yielding two amplicons in males and one in females, whereas in cluster kC72369, the 3' end of the reverse primer was targeted to a 3 bp male insertion to create a male-dominant marker.

Marker validation and loci characteristics

Primer pairs were first tested on a small set of male and female accessions of the corresponding species (see Supplemental information – Table S1) to confirm the verity of the GBS analysis prior to confirmation across the larger population. Following this initial confirmation, primer pairs designed within clusters aC36306, aC123485, and kC72369 were tested across the remaining accessions of known gender in the original, training populations (Supplemental information – Table S1). Primers for cluster kC48400 failed to amplify in accordance with the GBS results; so this cluster was dropped from further investigation. Both *A. arguta* primer pairs passed these initial screens and were further tested in two independent half-sib populations. Only one confirmed *A. kolomikta* primer pair, in cluster kC72369, was tested in an independent half-sib population. Ultimately, a primer pair from one cluster for each species, namely aC36306 and kC72369, displayed perfect sex linkage across all lines of their respective validation populations (Table 3).

The validated marker aC36306 amplifies a 106 bp band in *A. arguta* males with none to various alternate, presumably off-target fragments in females (Table 4; Supplemental information – File S1). When the aC36306 primers are multiplexed with a second universal forward primer nested between them, males are indicated by both an 81 bp and a 106 bp band, while females carry only a single 81 bp band (Figure 1, top). The validated marker kC72369 amplifies a 161 bp band in *A. kolomikta* males and a series of alternative loci in females (Table 4), with the brightest female band typically of a slightly higher molecular weight than the male band (Figure 1, bottom). Given the internal control included in the *A. arguta* marker and the consistent non-target amplification

TABLE 1. Summary of *de novo* variant calling using GBS-SNP-CROP, along with descriptions of putative sex-linked variants.

Species	Genotypes ^a	PE reads ^b	Total clusters ^c	Polymorphic clusters ^d	Sex-linked clusters ^e	Var. type ^f	Var. called ^g	Sex-linked var. ^h	Depth ⁱ	Hetero (%) ^j	Homo (%) ^k	NA (%) ^l
<i>A. arguta</i>	59	286,335,086	173,164	11,164	3	SNPs	30,468	2	59.99	27.82	60.17	11.99
						indels	1,754	1	64.58	24.42	65.15	10.41
						Total	32,222	3	—	—	—	—
						Mean	—	—	60.24	27.63	60.44	11.93
<i>A. kolomikta</i>	13	69,686,586	115,680	6,719	4	SNPs	12,067	6	30.53	35.20	58.16	6.64
						indels	419	2	38.29	39.25	58.86	6.89
						Total	12,486	8	—	—	—	—
						Mean	—	—	34.41	35.33	58.02	6.65

^a The number of accessions of known gender from each species.^b The total number of paired-end (PE) reads, considering all genotypes sampled.^c Total number of non-redundant consensus sequences (clusters) identified via clustering to represent the GBS fragment space used to build the reference sequence for alignment (i.e., the number of FASTA entries in the mock reference).^d The total number of polymorphic clusters.^e The number of clusters with putative sex-linked variants.^f The type of variants called, either single nucleotide polymorphism (SNP) or insertion-deletion (indel).^g The total number of variants called by the GBS-SNP-CROP pipeline.^h The number of putative sex-linked variants, by type.ⁱ Average read depth for all variants across the entire population.^j Percentage of heterozygous genotype calls.^k Percentage of homozygous genotype calls.^l Percentage of missing cells (i.e., no genotype call for a given SNP*accession combination) in the final SNP genotype matrix.**TABLE 2.** Details of the putative sex-linked variants (homozygous females, heterozygous males) identified via GBS association within the US collection of 60 *A. arguta* and 13 *A. kolomikta* genotypes.

Species	Cluster ID ^a	Length (bp) ^b	Var. type ^c	Var. position ^d	Female homo ^e	Male hetero ^f	Male homo ^g	NA ^h	Female genotype	Male genotype
<i>A. arguta</i>	70626 (1)	128	SNP	115	40	15	2	3	C/C	C/T
	36306 (1)	157	SNP*	64	40	13	1	6	T/T	T/C
	123485 (1)	131	indel*	94	36	12	4	8	A/A	A/-10GGTTTACCTT
<i>A. kolomikta</i>	693 (1)	43	SNP	32	10	3	0	0	T/T	T/G
			SNP	6	10	3	0	0	G/G	G/A
	1273 (3)	203	SNP	17	10	3	0	0	T/T	T/C
			indel	189	10	3	0	0	T/T	T/-2TG
	48400 (2)	221	SNP*	63	10	3	0	0	C/C	C/T
			SNP*	153	10	3	0	0	C/C	C/T
	72369 (2)	270	SNP	57	10	3	0	0	A/A	A/T
			indel*	154	10	2	1	0	G/G	G/+3ACC

^a The identification number of the GBS fragment (cluster/centroid) in the Mock Reference within which putative sex-linked variants were found. Numbers in parentheses indicate the number of variants identified within each cluster.^b The total length of the GBS cluster, in base pairs.^c The type of variants called, either single nucleotide polymorphism (SNP) or insertion-deletion (indel). An asterisk (*) indicates a variant around which primer pairs were designed.^d The variant position within the cluster, measured in bases from the start of the cluster sequence.^e The number of females scored as homozygotes. In this initial association analysis, there were a total of 42 and 8 female accessions for *A. arguta* and *A. kolomikta*, respectively.^f The number of males scored as heterozygotes. In this initial association analysis, there were a total of 17 and 3 male accessions for *A. arguta* and *A. kolomikta*, respectively.^g The number of males scored as homozygotes, potentially due to poor read depth. To be considered as potentially sex-linked, less than 5% of male lines were allowed to fall into this category.^h The number of lines in the populations for which genotypes could not be inferred, due to insufficient GBS read depth.

TABLE 3. Summary of PCR-inferred genders, relative to known genders, for lines in the initial populations as well as subsequent, independent validation populations for both species.

Initial populations														Validation populations													
Species	Marker ID	US Collection of <i>A. arguta</i> (<i>n</i> =59) ^a				OP progeny of 'Ogden Point' (<i>n</i> =17)				OP progeny of 'Hortgem Tahí' (<i>n</i> =21)				Fisher Exact <i>p</i> ^b													
		M	F	M _R	F _R	M	F	M _R	F _R	M	F	M _R	F _R														
<i>A. arguta</i>	aC36306	17	42	0	0	7	10	0	0	10	11	0	0	3.5×10 ⁻¹¹													
	aC123485	14	42	3	0	5	8	2	0	9	10	2	0		1.6×10 ⁻⁶												
Species	Marker ID	US Collection of <i>A. kolomikta</i> (<i>n</i> =13)								OP progeny of 'Nahodka' (<i>n</i> =21)				Fisher Exact <i>p</i> ^b													
		M	F	M _R	F _R					M	F	M _R	F _R														
<i>A. kolomikta</i>	kC72369	3	10	0	0					11	10	0	0	7.2×10 ⁻¹⁰													

^a Subscripted R's indicate recombination, or broken linkage, between the tested marker and the sex phenotype (e.g., F_R is a phenotypic female that amplifies a male band).

^b Fisher Exact Probability Test p-values for the validation populations.

TABLE 4. Primer and consensus GBS fragment (i.e., centroid) sequences for the validated, sex-linked markers in the two species *A. arguta* and *A. kolomikta*.

Species	Primer ID ^a	Primer sequence ^b	Tm ^c	Band size	Marker type
<i>A. arguta</i>	aC36306_male_for	TTTGGCAAAAACCACATg AC	52°C	106 bp	Male
	aC36306_univ_rev	AAAAGGGGGTTCGAAC TTTG			Dominant
	aC36306_univ_for	TGTTGGATGCCATTAAGTCG		81 bp	Internal
	aC36306_univ_rev	AAAAGGGGGTTCGAAC TTTG			Control
Cluster 36306 Paired Male and Female Sequences ^d					
♂	1	GTTAGCCAAAAAATGGTAATATGTTACATTGGTACAATATCATTTTGGC			
♀					
♂	51	AAAAACCACATCA C CTTTCTGTTGGATGCCATTAAGTCGAAGCCGTCCAT			
♀		T.....			
♂	101	TGGTTGAAACAAGTATTCCCACCCTTGGATCAAAGTTCGAACCCCTTTT			
♀					
♂	151	AGGTCCG			
♀					
<i>A. kolomikta</i>	kC72369_univ_for	TAGCCAAGGTAATGCCTCCA	52°C	161 bp	Male Dominant
	kC72369_male_rev	CAGGAATATGTTCAAGAGTT GGT			
Cluster 72369 Paired Male and Female Sequences					
♂	1	GCTTATGTTACCAAGCTAGCCAAGGTAATGCCTCCACCAACATTTTCTTT			
♀					
♂	51	ATATTTACACGTCTTAGTTTGATTATTTTTGTACAGAGACTTGACTCCGA			
♀		A.....			
♂	101	CCTTTAATTATTTTATTGGATATTCAAAGTGTCTCTGCATTTTCAGCT			
♀					
♂	151	GTAG ACCA ACTCTTGAACATATTCCTGGTCAAAATTATTCAATTTTCATAT			
♀					
♂	201	GCAATTCAATAGAAAATTACAAAGTTTTTAACTCAATGAGAGAGATTGTG			
♀					
♂	251	TAGATTACTTTTTATTCTGTCT			
♀					

^a Within the Primer IDs, "male" means the primer is male-specific, "univ" means the primer amplifies both male and female alleles, "for" = forward primer, and "rev" = reverse primer.

^b Nucleotides in bold are male-specific; lower-case nucleotides are degenerate, matching neither male nor female alleles.

^c Optimal annealing temperatures (Tm) were determined via temperature gradient trials.

^d Underlined sequences indicate the locations of the primers in the centroids; a dot (·) in the female-specific sequences indicates consensus with the male-specific base; a dash (-) in the female-specific sequences indicates a deletion, relative to the male-specific base.

in *A. kolomikta* females, both markers are effectively co-dominant, helping to reduce inaccurate scoring due to PCR failures. Neither marker was found to exhibit sex-linkage in the other species (data not shown).

When used as BLAST query sequences against the *A. chinensis* var. *chinensis* draft genome (Huang et al., 2013), the cluster sequences of both markers align to regions in the “Unknown” pseudomolecule, with the amplified regions of clusters aC36306 and kC72369 aligning at 97% and 92% identity, respectively. Neither marker aligns to known coding regions or possesses significant similarity to either repetitive or non-coding sequences in the NCBI database.

Discussion

As the quality and affordability of genome sequencing technologies continue to improve, a practical challenge facing marker-assisted breeders is how to use such data to efficiently identify trait-associated polymorphisms amenable to conversion into cost-effective molecular markers for screening large populations. This bioinformatic challenge of mining large genomic datasets for potential low-cost markers is amplified for breeders of minor and orphan crops due to there often being: 1) Little to no genomic resources available (e.g., high-quality reference genomes) to facilitate marker discovery; and 2) Considerable resource constraints compared to breeding programs for major crops. This study demonstrates the practical use of the GBS-SNP-CROP pipeline (Melo et al., 2016) to discover trait-linked polymorphisms (both SNPs and indels) in the absence of a reference genome. Candidate polymorphisms were converted into low-cost, gel-based PCR markers and validated in independent populations, thereby providing a concrete example of the successful use of *de novo* GBS analysis for marker development in minor crops, both diploid (*A. kolomikta*) and tetraploid (*A. arguta*).

Given their consistent homozygosity in females and heterozygosity in males, the markers developed and validated in this study support the ploidy-independent, Y-active sex inheritance system observed in *A. chinensis* var. *chinensis* and *A. chinensis* var. *deliciosa* (Gill et al., 1998; Fraser et al., 2009; Testolin et al., 1995, 1999). While breeding programs for these more globally important kiwifruit species have benefitted from the development of sex-linked markers for

many years (Harvey et al., 1997; Shirkot et al., 2002; Zhang et al., 2015), analogous markers for *A. arguta* and *A. kolomikta* have been lacking. The perfect sex-linkages of the two reported markers across the populations in this study suggest they may be used to confidently score progeny in cold-hardy kiwifruit breeding programs, resulting in a significant increase in resource efficiency. Specifically, given the slight male bias observed among populations of *A. arguta* progeny (Supplemental information – Table S2), the use of marker a36306 at the seedling stage should allow programs to effectively double the sizes of their breeding populations without driving up overall costs.

Due to the 2010 ban on the importation of *Actinidia* germplasm into the US to prevent the introduction of the pathogen *Pseudomonas syringae* pv. *actinidiae* (USDA, 2010), this study focused predominantly on lines within the US collection. While the relevance of these markers to other germplasm collections of *A. arguta* and *A. kolomikta* should be confirmed before they are adopted by non-US breeding programs, the validation of marker a36306 in the OP population from cv. Hortgem Tahi is encouraging. Not only is ‘Hortgem Tahi’ not in the US collection, its population of half-sib progeny likely represent even more non-US genetic diversity as they were derived from seeds of store-bought berries produced in and imported from New Zealand. If breeding programs discover that the markers reported here do not amplify in certain populations, the other sex-associated sequences discovered by this study (Supplemental information – File S1) may prove useful in fast-tracking the development of alternative markers, including CAPS markers.

Finally, it is worth noting that the clarity of gel-based markers depends not only on the inherent properties of the sequences involved, but also on the DNA extraction method and the PCR cycling conditions used. The method of DNA isolation used here sufficed for the development and validation of the reported markers, but PCR efficiency was seen to vary across batches. While amplification inhibition, when observed, was typically remedied through either a reduction of template (i.e., genomic DNA) concentration in the PCR reaction and/or an increase in cycle number, medium-to-high-throughout molecular screens require uniformity. To that end, potential users of these markers are

FIGURE 1. Agarose (2%) gel electrophoresis of the PCR products of *A. arguta* marker aC36306 with an 81 bp internal control (top) and *A. kolomikta* marker kC72369 (bottom). The relative faintness of the 81 bp band in the upper gel is inherent to the Y-active sex inheritance system of *Actinidia* spp. For tetraploid *A. arguta*, the male (Y) allele is expected to be present at a quarter of the dosage (i.e., one-fourth the brightness) of control (i.e., universal) alleles. Lad = 50 bp Gene Ruler; NTC = non-template control, ♀ = female, ♂ = male.

encouraged to collect young leaf tissue and invest effort in optimizing the extraction process under their specific laboratory conditions.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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SUPPLEMENTAL INFORMATION – TABLE S1. List of the 60 non-redundant accessions of *Actinidia arguta* and the 13 non-redundant accessions of *A. kolomikta* used for the initial screening of sex-linked variants.

No.	Genotype name	PI #	CACT #	DACT #	Gender reported	Gender observed	GBS barcode	PE reads	Variants called	SRA number
<i>Actinidia arguta</i>										
1	#211A	637808	169	272	M	M	CATAT	6401008	30173	SRR3234153
2	#211B	637809	170	271	–	F	CACCA	5959902	30175	SRR3234114
3	#74 Female	637804	164	326	F	F	ATCTGT	4478434	29507	SRR3234194
4	119-40	617156	108	202	F	F	TGCCGCAT	3857758	29020	SRR3234174
5	125-40	617157	109	204	F	–	GGCTTA	3554890	29366	SRR3234179
6	127-40	617142	92	206	M	F	TAGCGGAT	1087034	17411	SRR3234154
7	127-40	617163	120	205	M	M	CCTTGCCATT	4746724	29922	SRR3234196
8	74-32	617113	54	127	M	M	GGTGACATT	5488740	29999	SRR3234121
9	74-46*	–	–	–	M	M	GGAACGA	4387350	29503	SRR3234155
10	74-49	617103	43	208	F	F	CTCGCGG	4739038	30092	SRR3234197
11	74-52	–	–	–	M	M	AACGTGCCT	3936772	29447	SRR3234193
12	74-55	617114	56	132	F	F	TATCA	3337274	29293	SRR3234177
13	Ananasnaya	617104	44	220	F	F	CGTGTCA	3368520	28981	SRR3234178
14	Andrey	–	–	–	M	M	TATTCGCAT	4494836	29621	SRR3234159
15	Chang Bai Mountain 1	617110	50.001	212	F	F	GCTCCGA	397712	8016	SRR3234135
16	Chang Bai Mountain 2	617110	50.002	212	F	F	CTCGCGG	3433118	29174	SRR3234117
17	Chang Bai Mountain 3	617110	50.003	212	F	F	CAGATA	11533362	30487	SRR3234099
18	Chang Bai Mountain 4	617110	50.004	212	M	M	CCTTGCCATT	8735374	30356	SRR3234104
19	Chang Bai Mountain 5	617110	50.005	212	F	F	ACTGCGAT	1855156	25457	SRR3234111
20	Chico	667888	114	223	F	F	ACAACCT	4743214	29847	SRR3234161
21	Cornell female	–	–	–	F	F	GCCTACCT	3747072	29727	SRR3234113
22	DACT 123	617152/667991	104	123	F	F	CAAGT	5751718	30157	SRR3234158
23	DACT 213	637813	175	213	F	M	ACAGT	4723552	30036	SRR3234199
24	DACT 214	637812	174	214	F	F	AACTGG	5322144	30231	SRR3234139
25	DACT 216	637811	172	216	F	F	GTCGCCT	3793736	29231	SRR3234163
26	DACT 218	637810	171	218	F	F	TTGCTG	5227524	29912	SRR3234183
27	DACT 260	617168	139	260	F	F	TATGT	8100576	30118	SRR3234165
28	Dumbarton Oaks*	617135	84	225	F	F	GAATGCAATA	6049356	29825	SRR3234143
29	Early Cordifolia	–	–	–	F	F	ATAGAT	4634780	29835	SRR3234167
30	Flowercloud male*	–	–	–	M	M	ATTAT	1606094	23072	SRR3234109
31	Frenchman's Bay	617162	116	226	F	F	GGTGT	6368934	29962	SRR3234107
32	Geneva 1	617101	30	040/227	F	F	ATATAA	1651662	22389	SRR3234186
33	Geneva 3	617133	80	229	F	F	TCTTGG	6276020	30075	SRR3234144
34	Hardy Red	617107	47	230	F	F	AACGTGCCT	4646988	29359	SRR3234124

SUPPLEMENTAL INFORMATION – TABLE S1. (Continued).

No.	Genotype name	PI #	CACT #	DACT #	Gender reported	Gender observed	GBS barcode	PE reads	Variants called	SRA number
35	HVSC-115*	641101	186	231	F	F	ATGAGCAA	2840748	27899	SRR3234118
36	HVSC-117	641102	188	232	–	M	CAACCACACA	3209702	28933	SRR3234134
37	Issai	617106	46	233	F	F	GAAGCA	4525900	27956	SRR3234123
38	Issai small fruit variant	617116/667909	58	134	F	F	ATATCGCCA	3151572	27660	SRR3234120
39	Jumbo	617108	48	234	F	F	ATGAGCAA	5544624	30045	SRR3234146
40	Male Hardy Kiwi	–	–	–	M	M	CAAGT	4940840	29546	SRR3234125
41	Michigan State	617136	86	239	F	F	GGTGT	5521514	30052	SRR3234149
42	New Zealand	617125	70	334	F	F	TGGCAACAGA	965764	20792	SRR3234191
43	Ogden Point*	617140	90	240	F	F	AACGCACATT	2749792	28370	SRR3234136
44	Opitz	637803	163	241	M	M	GGTATA	5665492	30211	SRR3234105
45	Opitz Male*	637802	162	242	M	M	TCTTGG	6587636	30120	SRR3234106
46	ORUS 1-3	667903	254	244	–	M	GGAAGACAT	3857518	29431	SRR3234102
47	ORUS 1-4	667890	239	322	–	F	TGCTT	4645838	29675	SRR3234116
48	ORUS 1-5	667899	250	245	–	F	TCACTG	4455038	28879	SRR3234127
49	ORUS 1-6	667889	238	246	–	F	CATAT	11782074	30300	SRR3234130
50	ORUS 1-8*	667904	255	247	–	M	ACAGT	2096570	25534	SRR3234128
51	ORUS 2-1	667905	256	248	M	M	CTTGA	8998044	30153	SRR3234151
52	ORUS 2-16 [MR]	667891	241	249	F	F	TAGCGGAT	12638534	30824	SRR3234100
53	ORUS 2-17*	667901	252	250	–	M	GGATA	2219262	25952	SRR3234108
54	ORUS 2-3	667900	251	333	–	F	CTCTA	4351174	29586	SRR3234192
55	ORUS 2-7*	667895	245	252	–	F	CATCTGCCG	2135604	25584	SRR3234132
56	ORUS 3-3	667892	242	254	–	F	GGACAG	8156828	30153	SRR3234133
57	Red Princess	617118	60	274	F	F	AATAACCAA	3386808	29338	SRR3234122
58	Smith 2 Female	617130	75	255	–	–	TGGCACAGA	4624310	29932	SRR3234176
59	Tatyana	–	–	–	F	F	ATATAA	1469580	23415	SRR3234112
60	West*	617121	66	257	F	F	GCGTCCT	6337356	29702	SRR3234156
<i>Actinidia kolomikta</i>										
1	A-10*	617145	95	275	F	F	GTGACACAT	7989260	12078	SRR3234080
2	Ananasnaya	–	–	–	F	F	AAGACGCT	4765536	11872	SRR3234096
3	Dr. Szymanowski	617166	137	280	F	F	GGCTTA	5907658	11703	SRR3234072
4	Frost	–	–	–	F	–	CGCACCAATT	5687430	12086	SRR3234093
5	Klara Zetkin*	–	–	–	F	F	ATATCGCCA	3464698	11699	SRR3234091
6	Krupnoplodnaya	617122	67	282	F	M	ATCTGT	3596006	10868	SRR3234071
7	Hero	–	–	–	F	–	TCACGGAAG	6626818	11587	SRR3234085
8	Leningradskaya ozdnyaya*	617150	101	285	M	M	CGTTCA	5341386	12025	SRR3234097
9	Matovnaya [MR]	617147	97	286	F	F	CAGTGCCATT	9769392	12120	SRR3234083
10	Nahodka	617120	65	287	F	F	TTCGTT	2343654	10159	SRR3234075
11	Oluyckos*	641095	127	289	F	F	CGTGGACAGT	4791980	11982	SRR3234084
12	Raintree*	617126	71	292	M	M	TCACTG	5763436	11940	SRR3234094
13	Urozhnaya	641096	129	295	–	F	GAGCGACAT	3639332	11426	SRR3234073

For each accession, the following information is provided:

- 1 Accession name (* = genotypes used for initial PCR-based marker testing; [MR] = the female used to build each species-specific Mock Reference, due to its high number of PE reads).
- 2 USDA Plant Introduction (PI) number(s), if assigned.
- 3 USDA Corvallis *Actinidia* (CACT) accession number, if assigned.
- 4 USDA Davis *Actinidia* (DACT) accession number(s), if assigned.
- 5 Reported gender.
- 6 Gender observed at UNH (Note: In cases of conflict between the reported gender and the gender observed at UNH, the observed gender of the plant from which DNA was isolated was used).
- 7 GBS barcode assignment.
- 8 Number of high-quality paired-end (PE) reads used to call SNPs.
- 9 Number of variants (SNPs and indels) called.
- 10 Assigned NCBI Sequence Read Archive (SRA) number.

SUPPLEMENTAL INFORMATION – TABLE S2. Genotype-inferred genders of full-sib progeny from 13 *A. arguta* crosses, based on the sex-linked marker *aC36306*.

Population (mother × father)	Males	Females	% Males
Dumbarton Oaks × Russia #114	19	11	63.3
UCD 3-1 × CACT 160 #111	47	17	73.4
Dumbarton Oaks × Chang Bai Mountain 4	5	5	50.0
Tatyana × 74-32	23	16	59.0
Ogden Point × CACT 105 #107	74	54	57.8
Ogden Point × C-5 (HRC)	88	74	54.3
UCD 3-1 × C-5 (HRC)	19	22	46.3
Dumbarton Oaks × C-5 (HRC)	56	65	46.3
Chang Bai Mountain 5 × 74-32	61	53	53.5
Dumbarton Oaks × CACT 105 #107	68	60	53.1
DACT 218 × CACT 105 #107	20	15	57.1
Ogden Point × Chang Bai Mountain 4	68	75	47.6
Ogden Point × CACT 160 #111	65	51	56.0
Totals	613	518	
Mean			55.2

SUPPLEMENTAL INFORMATION – FILE S1. Paired FASTA entries describing the nucleotide sequences of homozygous female and heterozygous male genotypes for putative sex-linked variants, based on the initial GBS association analyses.

FASTA header structure:

>GBS cluster ID within the Mock Reference | Cluster length (bp) | Polymorphism type | Mock Reference allele | Polymorphism position within the cluster (bp) | Female/Male genotype.

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>Cluster70626|128|SNP|C|115|Female (C/C)
1      GTGATACTTGAGCGAGTAAGCTTCTCCAAGGAGCCAAGTGATCCCCCTCCA
51     AGGAGCCAAGAGGTCCCCTCTAAGTGATCGCCTCCAAGGAGCTCCCCCTCC
101    AACAGGTTCCCCCTTCAAAGAGCTCCCCG
>Cluster70626|128|SNP|C|115|Male (C/T)
1      GTGATACTTGAGCGAGTAAGCTTCTCCAAGGAGCCAAGTGATCCCCCTCCA
51     AGGAGCCAAGAGGTCCCCTCTAAGTGATCGCCTCCAAGGAGCTCCCCCTCC
101    AACAGGTTCCCCCTTCAAAGAGCTCCCCG

>Cluster36306|157|SNP|T|64|Female (T/T)
1      GTTAGCCAAAAAATGGTAATATGTTACATTGGTACAATATCATTTTGGC
51     AAAAACCACATCACTTTCTGTTGGATGCCATTAAGTCGAAGCCGTCAT
101    TGGTTGAAACAAGTATTCCCACCCTTGGATCAAAGTTCGAACCCCTTTT
151    AGGTCCG
>Cluster36306|157|SNP|T|64|Male (T/C)
1      GTTAGCCAAAAAATGGTAATATGTTACATTGGTACAATATCATTTTGGC
51     AAAAACCACATCACTTTCTGTTGGATGCCATTAAGTCGAAGCCGTCAT
101    TGGTTGAAACAAGTATTCCCACCCTTGGATCAAAGTTCGAACCCCTTTT
151    AGGTCCG

>Cluster123485|131|Indel|A|94|Female (A/A)
1      GTTCTGAGCAGCCAAGCAGTTGGAACCAAGAAAAAATTCACCCAGTAA
51     CTAATCAAATTCTTCTAAACAAAAACGAGATTGAAGAATTGAAGGGTTA
101    CCTTGAGTATGAGATCGTCTAGGCGTCGCCG
>Cluster123485|131|Indel|A|94|Male (A/-10GGGTTACCTT)
1      GTTCTGAGCAGCCAAGCAGTTGGAACCAAGAAAAAATTCACCCAGTAA
51     CTAATCAAATTCTTCTAAACAAAAACGAGATTGAAGAATTGA-----
101    -----GAGTATGAGATCGTCTAGGCGTCGCCG

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SUPPLEMENTAL INFORMATION – FILE S1. (Continued).*Actinidia kolomikta*

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>Cluster693|43|SNP|T|32|Female (T/T)
1      GTATTATTATTGGGTGGGCTATGCCCAAAGGTCCGGGGAAAGA
>Cluster693|43|SNP|T|32|Male (T/G)
1      GTATTATTATTGGGTGGGCTATGCCCAAAGGGCCGGGGAAAGA

>Cluster1273|203|SNP|G|6|Female (G/G)
>Cluster1273|203|SNP|T|17|Female (T/T)
>Cluster1273|203|Indel|T|189|Female (T/T)
1      GGTACGGCTGTTATGATGTCCAGAGGGTGGTTTTTGCAATAATTGCAGAC
51     TTTCTCCTTAGAGCAGCTCAGATTGCGAGTGCAGTACAAAGGGAAAATGG
101    CACGTTGTAGAAACAATTCCTGAAGCCTGAAGGGTCTAAAAACAATATAA
151    GTATTGATGAGCTCCGAAACAAGTTTGAGACGTTGCATTGTGCCCCGGG
201    GAA
>Cluster1273|203|SNP|G|6|Male (G/A)
>Cluster1273|203|SNP|T|17|Male (T/C)
>Cluster1273|203|Indel|T|189|Male (T/-2TG)
1      GGTACAGCTGTTATGACGTCCAGAGGGTGGTTTTTGCAATAATTGCAGAC
51     TTTCTCCTTAGAGCAGCTCAGATTGCGAGTGCAGTACAAAGGGAAAATGG
101    CACGTTGTAGAAACAATTCCTGAAGCCTGAAGGGTCTAAAAACAATATAA
151    GTATTGATGAGCTCCGAAACAAGTTTGAGACGTTGCAT--TGCCCCGGG
201    GAA

>Cluster48400|221|SNP|C|63|Female (C/C)
>Cluster48400|221|SNP|C|153|Female (C/C)
1      GGTTGCATGCATGCAGAATCAACATCACACAGCAATTGCACAAGTCAATA
51     TAACCCTAGATTCGACAAATAAACTTCATTTTTTTTAATTGAAGACGGGT
101    CATTGGCAGGATGCAGCTCGAAGCAGGCTCGAGGTAAATGACAATATTA
151    AGCAGTAACTCAGATGTGTAAATGACAGTCAGTAAAAGCCGAAGTACGAA
201    AAGCAGAAATTTACCGGGGAA
>Cluster48400|221|SNP|C|63|Male (C/T)
>Cluster48400|221|SNP|C|153|Male (C/T)
1      GGTTGCATGCATGCAGAATCAACATCACACAGCAATTGCACAAGTCAATA
51     TAACCCTAGATTTGACAAATAAACTTCATTTTTTTTAATTGAAGACGGGT
101    CATTGGCAGGATGCAGCTCGAAGCAGGCTCGAGGTAAATGACAATATTA
151    AGTAGTAACTCAGATGTGTAAATGACAGTCAGTAAAAGCCGAAGTACGAA
201    AAGCAGAAATTTACCGGGGAA

>Cluster72369|270|SNP|A|57|Female (A/A)
>Cluster72369|270|SNP|G|154|Female (G/G)
1      GCTTATGTTACCAAGCTAGCCAAGGTAATGCCTCCACCAACATTTTCTTT
51     ATATATACAGTCTTAGTTTGATTATTTTTGTACAGAGACTTGACTCCGA
101    CCTTTAATTATTTTATTGGATATTCAAAGTGTCCTCTGCATTTTCAGCT
151    GTAC---AACTCTGAACATATTCCTGGTCAAAATTAATCAATTTTCATAT
201    GCAATTCAATAGAAAATTACAAAGTTTTTAACTCAATGAGAGAGATTGTG
251    TAGATTACTTTTTATTCTCTG
>Cluster72369|270|SNP|A|57|Male (A/T)
>Cluster72369|270|SNP|G|154|Male (G/+3ACC)
1      GCTTATGTTACCAAGCTAGCCAAGGTAATGCCTCCACCAACATTTTCTTT
51     ATATATTCAGTCTTAGTTTGATTATTTTTGTACAGAGACTTGACTCCGA
101    CCTTTAATTATTTTATTGGATATTCAAAGTGTCCTCTGCATTTTCAGCT
151    GTAGACCAACTCTGAACATATTCCTGGTCAAAATTAATCAATTTTCATAT
201    GCAATTCAATAGAAAATTACAAAGTTTTTAACTCAATGAGAGAGATTGTG
251    TAGATTACTTTTTATTCTCTG

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