GENETIC AND ENVIRONMENTAL CONTROL OF STEVIOL GLYCOSIDE BIOSYNTHESIS IN STEVIA REBAUDIANA

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ABSTRACT

GENETIC AND ENVIRONMENTAL CONTROL OF STEVIOL GLYCOSIDE BIOSYNTHESIS IN STEVIA REBAUDIANA

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Stevia rebaudiana (stevia) is gaining agricultural importance due to its natural production of non-caloric steviol glycosides, which can be extracted and incorporated into natural, zero-calorie sweeteners. Of the several steviol glycosides produced by stevia, those produced in the largest concentrations, such as rebaudioside A, are often criticized by consumers as having a bitter aftertaste. Other glycosides, such as rebaudioside D, are reported to have a superior flavor profile, but are produced in much smaller concentrations. The environmental and genetic control of steviol glycoside synthesis is poorly understood. The objectives of this research were: 1) to determine how daily light integral (DLI) influences glycoside concentrations and relative proportions, particularly for rebaudioside D and 2) to elucidate the genetic control of rebaudioside D synthesis from rebaudioside A. DLI influenced both glycoside concentration and relative proportion in a glycoside-specific manner. Using traditional breeding, populations were created via controlled crosses where parents varied in the percentage of rebaudioside D calculated from the total of rebaudiosides A and D. The production of rebaudioside D appears to be regulated by a single dominant gene controlling the presence/absence of the precursor compound, rebaudioside A. Some of the individual populations in this study exhibited correlations unique to each population regarding traits of interest, demonstrating the variability in stevia. Future breeding efforts could harness this variability in addition to knowledge of precursor glycoside content as a resource to improve future stevia lines.

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"Gratitude is the memory of the heart." – Jean Baptiste Massieu

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CHAPTER ONE

LITERATURE REVIEW

Introduction to *Stevia rebaudiana*

The genus *Stevia* currently has 220-230 species, with one species of economic importance, *S. rebaudiana* (Kinghorn, 2002). What distinguishes *S. rebaudiana* (stevia) is a relatively high production of non-toxic, non-nutritive ent-kaurene diterpenoid glycosides in the leaves, some of which are approximately 300 times sweeter than sucrose (Yadav et al., 2011). The appeal for producing, harvesting and extracting select glycosides follows a consumer desire for a natural, zero-calorie sweetener. Additional benefits of consuming stevia products include healthy blood pressure regulation, antiviral and antimicrobial properties for immune health, appropriate glucoregulation for consumers afflicted with diabetes, and prevention of dental cavities (Tarka & Roberts, 2010)

Stevia rebaudiana is a New World crop, endemic to Paraguay as originally classified by Bertoni, and confirmed by Sumida in 1975 (Kinghorn, 2002). A member of the Eupatorieae tribe in the Asteraceae family, it has been used by the native Guarani Indians for centuries to sweeten bitter foods and medicines (Ramesh et al., 2006). Naturally possessing a slender, herbaceous growth habit, *S. rebaudiana* naturally occrus around marshes or grasslands with shallow water tables. Often, the soils are infertile, acidic, sand or muck types, continuously moist but not subject to inundation for prolonged periods of time (Shock, 1982). A perennial in its native environment, cultivated stevia can be grown as an annual or perennial (Ramesh et al., 2006).

Stevia rebaudiana is diploid, with eleven chromosomes. Many other species of *Stevia* share this characteristic, but have distinguishable karyotypes, due to the gain or loss of chromatin during species divergence (Frederico & Ruas, 1996). Polyploidy is documented within *S. rebaudiana*, but this arose by artificial induction to develop lines with high levels of stevioside

(Oliveira et al., 2004). Stevia is widely reported as self-incompatible and most is likely insect-pollinated (Ramesh et al., 2006). Fertile seeds have been harvested from self-pollinated plants (Jeffrey Goettemoeller & Ching, 1999), but the amount of seed was not reported, nor were the progeny verified to be selfs. Overall, stevia is highly outcrossing, which challenges breeding efforts by preventing the creation of inbred lines. Conversely, high heterozygosity provides variation as a resource for improvement.

The scientific study of *Stevia rebaudiana* was initiated by Moises Santiago de Bertoni and Ovidio Rebaudi. Bertoni named the species *Stevia rebaudiana* in 1905, dedicating the species name to Rebaudi, the chemist who first studied the sweet compounds (Bertoni, 1905, 1918). In 1899 Bertoni had assigned an incorrect name (*Eupatorium rebaudianum)* to a plant sample given to him by a Paraguayan customs officer intended to sweeten his bitter drink (maté). It wasn't until Bertoni received a live specimen that he was able to determine the correct taxonomic placement of *S. rebaudiana* (Kinghorn, 2002).

Commercial use of stevia extracts as food additives first began in Japan following a consortium created to discuss the potential use of stevioside (Yadav et al., 2011). Japan has been using *S. rebaudiana* as a sweetening agent since the mid-1970's following a temporary ban on sodium saccharin. The Japanese stevia sweetener market has expanded and is now valued at two to three billion yen (19.4 – 29 million US dollars) per year, and is used to flavor over 25% of Japanese pickles (Kinghorn, 2002). Following Japan with approval in 1984, the Korean sweetener market favors the use of stevioside and has increased to occupy 40% of the market. The production of the Korean sweet potato liquor soju demanded half of the total share, ca.116 metric tons in 1995. As in Japan, much of the raw leaf material is imported from China (Kinghorn, 2002). In addition to Japan and Korea, Brazil has also allowed stevioside and stevia

extracts as food additives. The United States F.D.A. accepted high purity rebaudioside A as "Generally Recognized as Safe" in 2008. The European Union followed with approval of the use steviol glycosides as sweeteners in foods and beverages in 2011. In more recent years, there has been increasing focus on other glycosides due to their increased palatability. Rebaudioside D is reported to have less "astringency, bitterness, numbing and licorice" taste perception attributes than rebaudioside A at the same concentration, and behaves more like sucrose in terms of sweetness properties (Lee et al., 2013). Rebaudioside X shows improved flavor characteristics when compared to both rebaudiosides A and D (Prakash et al., 2013). Rebaudioside X has been described as having "a clean, sweet taste with a broad and full sweetness profile… much better temporal profile than rebaudiosides A or D, and quality similar to sucrose." Rebaudioside X also has a similar sweetness profile at room temperatures and 4° C, whereas rebaudiosides A and D are significantly more sweet at 4° C (Prakash et al., 2013).

Effect of Light Environment on Steviol Glycosides in *Stevia rebaudiana*

Environmental factors such as photoperiod (Metivier & Viana, 1979; Yermakov & Kochetov, 1996) and irradiance (Ermakov & Kochetov, 1994; Yermakov & Kochetov, 1996) can have a large impact on plant phenotype in addition to its genetic composition. Total concentrations of glycosides can vary from year to year, whereas the proportions of individual accumulated glycosides remain similar, which has promoted the use of proportions to make comparisons between populations (Brandle, 1998).

Day length can affect levels of stevioside produced by *Stevia rebaudiana.* Metivier and Viana (1979) found that stevioside levels in plants exposed to long days (LD; 16 h) were over four times higher than plants grown under short days (SD; 8 h). This study also compared the partitioning of soluble sugars and steviol between LD and SD treatments. However, the LD and

SD treatments supplied the same amount of instantaneous irradiance. Therefore, photoperiod effects were confounded by differences in daily light integral (DLI), or the amount of photosynthetic light received per day. Stevioside concentrations were 72 μg mg^{-1} for LD and 37 μg mg^{-1} for SD, with the LD plants allocating 40% of the soluble sugar pool to stevioside, compared to about 33% in the SD treatment. Plants grown under LD synthesized 0.16 μg of steviol for every μg of soluble sugar, whereas the short day plants averaged only 0.11 μg per μg of soluble sugar, suggesting that photoperiod could have an influence on partitioning in this biosynthetic pathway (Metivier &Viana, 1979). This work only quantified stevioside and steviol levels over two photoperiods and did not examine important steviol glycosides of current interest such as rebaudiosides A, C, D or X.

Zaidan et al. (1980) compared three genotypes grown under an 8-h (SD) or 16-h (LD) photoperiod. Both photoperiods were comprised of 8 h of natural light, with the LD treatment extending the photoperiod with incandescent bulbs (4.8 μmol·m^{-2}·s^{-1}). For all genotypes, the concentration of stevioside was higher in LD plants. The authors also noted that the presence of flowers did not affect the amount of stevioside in the leaves. Another component of the study compared plants grown in SD greenhouse conditions to plants grown in SD natural conditions and quantified their stevioside levels. Stevia plants grown under natural conditions contained 375% more stevioside than the greenhouse grown plants. This led the authors to state that "the content of stevioside is related to the total irradiance received by the plant instead of the photoperiod itself" since plants grown outside almost certainly received higher irradiance, although this was not documented. However, the environmental conditions for the comparison were not replicated, raising questions about other environmental pressures endured by the plants grown in natural conditions. In that stevioside has been proposed to be produced as a defense

mechanism against herbivory (Zaidan et al., 1980) and the influence of irradiance is still an open question.

Mohamed et al. (2011) measured transcript levels of genes thought to be contributing to biosynthesis of steviol glycosides. The accumulation of total steviol glycosides was higher in plants kept under 8 h SD compared to 16 h LD, in contrast to results reported by Metivier and Viana (1979). Also, the enzymes catalyzing downstream reactions in the steviol glycoside pathway were more highly expressed in the SD grown plants. This experiment was not designed to look at environmental effects and it is unclear whether or not irradiance levels were standardized. Regardless, it does raise the question whether or not other steviol glycosides, particularly Rebaudiosides A, C, D and X follow the same trend as stevioside when exposed to different light periods.

Photoperiod can affect synthesis of secondary metabolites and many studies have explored the effects of photoperiod on steviol glycoside accumulation in *S. rebaudiana* (Ceunen & Geuns, 2013a; Metivier & Viana, 1979; Valio & Rocha, 1977; Zaidan et al., 1980). The effects of light quality, specifically red light (Ceunen & Geuns, 2012; Ceunen et al., 2012) have also been examined in stevia. Research pertaining to the amount of photosynthetic light received per day, (DLI), has been restricted thus far to quantifying only biomass for a range of DLIs under long days, with no reports of DLI effects on glycoside accumulation. (Ermakov & Kochetov, 1994; Yermakov & Kochetov, 1996). Kumar et al., 2013 found that a 25% light reduction significantly increased rebaudioside A levels in the developmental stage described as "flower initiation". Steviol glycoside concentrations during other stages were not affected by light reduction, however the study only reported on stevioside and rebaudioside A, leaving DLI effects on rebaudiosides B, C, D and X uncharacterized. No previous reports have focused on

glycosides beyond stevioside and rebaudioside A, therefore how the steviol biosynthesis pathway is affected by DLI is not well understood.

Genetic Control of Steviol Glycoside Biosynthesis

Stevia rebaudiana produces at least 34 steviol glycosides that can be isolated from leaf material (Ceunen & Geuns, 2013b). These compounds are synthesized through a series of enzymatic reactions, and genes encoding some of the enzymes catalyzing these reactions have been identified. The steviol glycoside pathway (Figure 1.1) shares common precursors with gibberellins in tissues that either have or have had chloroplasts. The hydroxylation of kaurenoic acid by 13-hydryoxylase (KAH) to form steviol is the first committed step in steviol glycoside synthesis. Glycosylation reactions are mediated by UDP- glycosyltransferases (UGTs), and begin following the exportation of steviol to the cytosol (Brandle and Telmer, 2007). Activated sugars such as UDP-glucose, are transferred to acceptor molecules by UGTs, resulting in a mixture of glycosides in leaf tissue of S. *rebaudiana* (Richman et al., 2005).

Glycosylated steviol is translocated to the vacuole from the cytosol, although how this occurs is still unclear (Brandle and Telmer, 2007). The transfer of these activated sugars to receptor molecules cause detoxification, stabilization, and solubilization of secondary metabolites (Richman et al., 2005). UGTs are not specific to *S. rebaudiana* and play a large role in the diversity of secondary metabolites that characterize the plant kingdom (Richman et al., 2005).

Twelve UGTs were examined for their ability to transfer an activated sugar to various substrates (Richman et al., 2005). Three of the twelve showed an involvement in steviol glycoside biosynthesis, UGTs 85C2, 74G1 and 76G1. The expression levels in leaves for these

three were not significantly higher than any of the other nine UGTs studied, with higher levels of expression in leaves compared to roots. Among the functionally characterized UGTs (UGT85C2, UGT74G1 and UGT76G1), several amino acid residues are conserved, which could be related to substrate specificity (Richman et al., 2005). UGT85C2 is associated with the conversion of steviol to steviolmonoside (Figure 1.1). Steviolmonoside is converted to rubusoside via UGT74G1, which is the UGT also responsible for catalyzing steviolbioside to stevioside, stevioside to rebaudioside E and also rebaudioside B to rebaudioside A (Mohamed et al., 2011). UGT76G1 catalyzes the conversion of stevioside to rebaudioside A (Richman et al., 2005a), and possibly the synthesis of rebaudioside C from dulcoside A (Brandle, 1999). Information regarding the genetic control of the biosynthesis of rebaudiosides D and X has yet to be validated *in vivo*, however, glycosylation of rebaudioside A to rebaudioside D has been demonstrated *in vitro*, mediated by the enzyme UGT91D2 (Kishore et al., 2011).

The accumulation of steviol glycosides is known to be affected by the method of propagation, day length (Metivier and Viana, 1979), and agronomic practices such as irrigation and fertilization (Shock, 1982). Environmental factors have been shown to influence UGT expression. For example, Mohamed et al., (2011) found that expression of UGT74G1 was much higher in the upper leaves under SD (12-h days) conditions compared to LD (16-h days) conditions, while the transcription of UGT76G1 and UGT85C2 remained unchanged. UGT 76G1 had higher levels of expression in upper leaves compared to lower leaves under LD. Steviol glycosides were found in higher concentrations in upper leaves compared to lower leaves under both LD and SD condtions, and the ratios of the glycosides seemed to be affected by day length. A significant correlation between UGT 85C2 transcription and total steviol glycosides was found in upper leaves under SD conditions. Rebaudioside A was found to be synthesized in

a 2:1 ratio with stevioside until placed in a SD environment which caused the ratio to decrease to 1:1 over time. This result is confounded by the light treatments themselves, since plants in the LD treatment may have received greater amounts of irradiance than their SD counterparts, as the method of day extension was not specified.

Classical breeding approaches have also increased understanding of the basis of steviol glycoside synthesis. Brandle (1999) concluded, after evaluating progeny from parents with dissimilar glycoside profiles (both F_1 and F_2 individuals), that the presence/absence of rebaudioside A is controlled by a single dominant gene, and hypothesized that the proportions of rebaudioside A are controlled by multiple alleles or loci. Rebaudioside C was also found to match the segregation of rebaudioside A, and the two were positively correlated. Since rebaudiosides A and C were found to cosegregate, and also because their respective precursor molecules, stevioside and dulcoside A, are structurally similar, the hypothesis that synthesis of rebaudiosides A and C could be catalyzed by the same enzyme was proposed (Brandle, 1999), and reflects our current understanding of the pathway (Figure 1.1).

Stevioside concentration, leaf:stem ratio, leaf yield and height have been shown to be highly heritable (Brandle and Rosa, 1992). The experimental design attempted to account for genotype by environment interactions by staggering the planting date by two weeks (May 1 and May 15). This adjustment may or may not be adequate to simulate multiple environments. Analysis of thirteen half-sib families identified significant variation for all traits except height. The fact that stevioside concentrations were not correlated to leaf:stem ratio or leaf yield suggests these traits can be improved for greater steviol glycoside yield via breeding efforts concomitantly (Brandle and Rosa, 1992).

Both classical breeding and molecular approaches have generated useful information for stevia improvement. Our understanding of genetic and environmental control of steviol glycoside biosynthesis is developing but still incomplete. Information relating to production of rebaudioside D is lacking and generally not represented in published literature. As mentioned previously, rebaudiosides D and X offer improved flavor perception compared to rebaudioside A. Therefore, processors are interested in increasing rebaudioside D and X concentrations produced by stevia. However, rebaudiosides D and X are synthesized at much lower concentrations than rebaudioside A. Therefore, understanding the environmental and genetic control of rebaudioside D synthesis is critical for developing improved varieties that can produce more glycosides of interest. Additionally, since rebaudioside D is structurally similar to and likely a precursor to rebaudioside X, information about rebaudioside D could lend to improved understanding of the synthesis of rebaudioside X.

APPENDIX

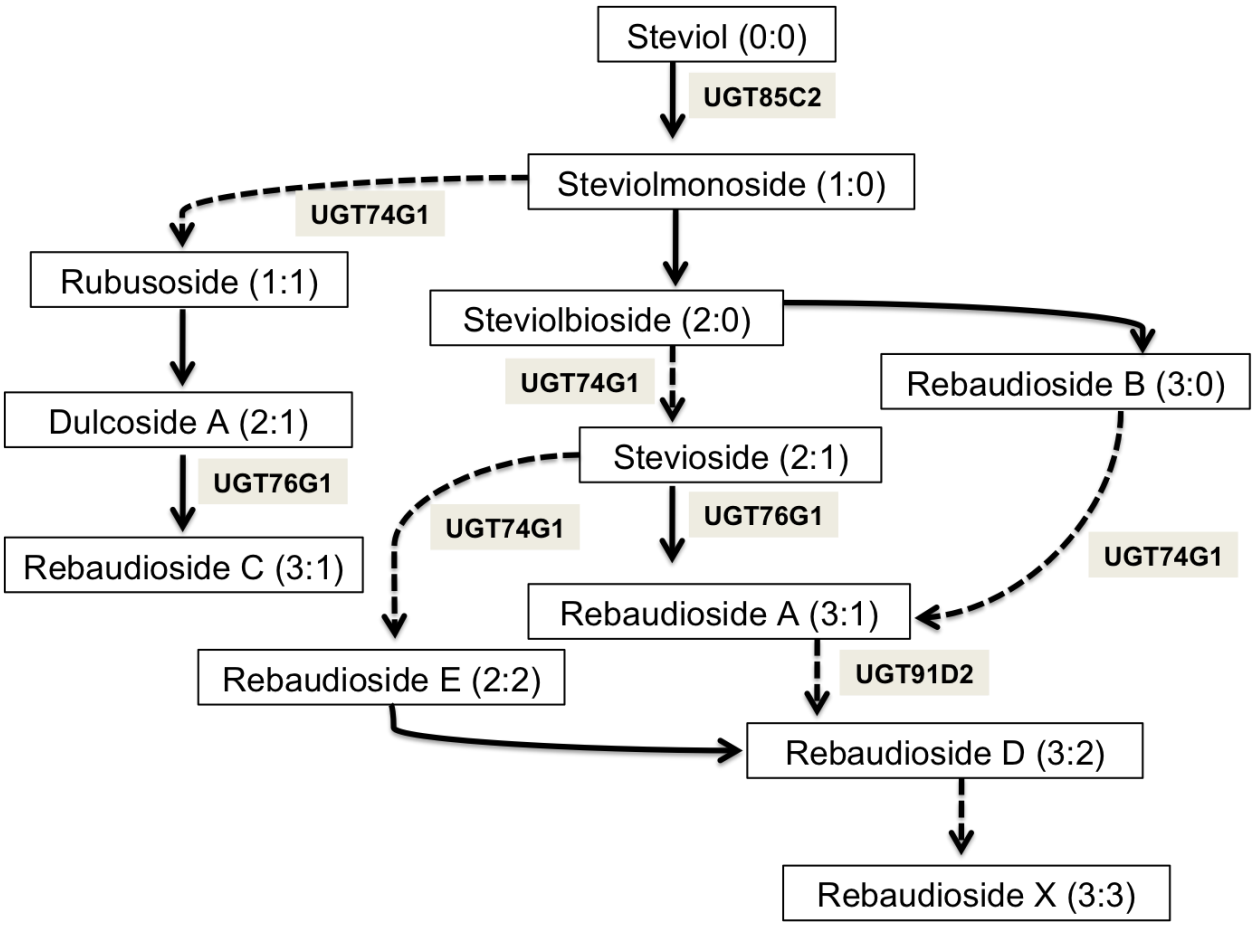

Figure 1.1. Excerpt of steviol glycoside biosynthesis pathway and known UDP-glycosyltransferases (UGTs), responsible for glycosylation events. Solid and dashed lines indicate C_{13} and C_{19} glycosylations, respectively. Values in parenthesis following compound name refer to pattern of glycosylated steviol (hexose sugar units attached to C_{13}: glucose units attached to C_{19}). Adapted from (Brandle, 1999; Kishore et al., 2011; Mohamed et al., 2011; Richman et al., 2005; Shibata et al., 1991).

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CHAPTER TWO

THE INFLUENCE OF DAILY LIGHT INTEGRAL ON STEVIOL GLYCOSIDE BIOSYNTHESIS

Introduction

The production of steviol glycosides in plant species is rare, a characteristic that *Stevia rebaudiana* shares with only three other known species, *Stevia phlebophylla* (Kinghorn et al., 1984), *Rubus sauvissimuss* (Ohtani et al., 1992) and *Angelica keiskei* (Ceunen & Geuns, 2013b). Steviol glycosides are tetracyclic diterpenes, which share precursors with gibberellins (Brandle & Telmer, 2007). Discovery of new diterpene glycosides has been swift in recent years, and as of April 2013, 34 steviol glycosides have been identified in *S. rebaudiana* (Ceunen & Geuns, 2013b).

The diversity of steviol glycosides results from differential glycosylation on the aglycone moiety, steviol, by several cytosolic UDP-glycosyltransferases (Brandle & Telmer, 2007). These UDP-glycosyltransferases, known as UGTs, transfer activated sugars, usually UDP-glucose, to acceptor molecules, which produces the mixture of glycosides found in leaf tissue of *S. rebaudiana* (Richman et al., 2005). The vacuoles of leave tissue contain stevioside, a tri-glycoside, and rebaudioside A, a tetra-glycoside, comprising the majority of glycosides found, however rebaudioside B (tri-glycoside), rebaudioside C (tetra-glycoside), rebaudioside D (penta-glycoside) and rebaudioside X (hexa-glycoside) can also be found in lesser amounts.

UGTs are not specific to *S. rebaudiana* and play a large role in the diversity of secondary metabolites that characterize the plant kingdom (Richman et al., 2005). As many as 12 UGTs have been detected in *S. rebaudiana* but only three have been linked to steviol glycoside synthesis, while a fourth UGT remains a candidate (Ceunen & Geuns, 2013b; Richman et al., 2005). How these multiple glycosylations are regulated is poorly understood, although some modes have been proposed, such as glycosylation of steviol by UGT85C2, the first step in steviol glycosides formation, to form steviolmonoside (Mohamed et al., 2011).

Many studies have explored the effects of photoperiod on steviol glycoside accumulation in *S. rebaudiana* (Ceunen & Geuns, 2013b; Metivier & Viana, 1979; Valio & Rocha, 1977; Zaidan et al., 1980) and the effects of light quality, specifically red light (Ceunen & Geuns, 2012; Ceunen et al., 2012). However, limited research has been performed regarding the effects of the daily light integral (DLI), or the amount of photosynthetic light received per day. Previous studies have quantified biomass yielding potential under a range of DLIs under long days, however no glycoside data was reported (Ermakov & Kochetov, 1994; Yermakov & Kochetov, 1996). A more recent study found that a reduction in light did not significantly affect steviol glycoside concentrations except at the crop development period described as the flower initiation stage, where rebaudioside A levels were significantly higher with 25% light reduction. However only stevioside and rebaudioside A levels were reported in this study, and no other glycosides were mentioned (Kumar et al., 2013). The effects of DLI on synthesis of rebaudiosides B, C, D, and X have not previously been reported. The objective of this study was to evaluate the influence of DLI on the concentrations and relative proportions of several steviol glycosides.

Materials and methods

Experiment 1

Plant material and propagation. On 14 February 2012 (run 1) and again on 24 July 2012 (run 2), two *Stevia rebaudiana* selections from an open-pollinated population derived from 'Morita II', designated 10-43-41 and 11-464, were vegetatively propagated into plug trays [128-cell size (12 ml volume)] in a soilless medium consisting of 50% fine perlite (Perlite Vermiculite Packaging Industries, Inc. North Bloomfield, OH, USA) and 50% potting mix composed of peat, perlite, lime and starter fertilizer (Suremix Perlite, Michigan Grower Products Inc., Galesburg,

MI, USA). The cuttings were rooted in a glass-covered greenhouse under a 16-h photoperiod [ambient daylight plus day-extension lighting provided by high-pressure sodium (HPS) lamps from 0600 to 2200 HR] at an air temperature of 24 °C and bench temperature of 26 °C. Bench temperature was regulated by hot water running through pipes inlayed directly under bench. The watering regime consisted of intermittent overhead mist supplemented with a water-soluble fertilizer providing (mg L^{-1}): 50 N, 8 P, 42 K, 22 Ca, 12 Mg, 1.0 Fe and Cu, 0.5 Mn and Zn, 0.3 B, and 0.1 Mo (MSU Prop Special, GreenCare Fertilizers, Inc., Kankakee, IL, USA). When plant roots reached all corners of the plug cell, rooted cuttings were transplanted into 10-cm round plastic containers (480 mL volume) filled with 100% Suremix Perlite potting mix and moved to the greenhouse environment described below.

Greenhouse environment and plant culture. Plants were grown under a photoperiod of 16 h (0600 to 2200 HR) achieved by supplementing the natural photoperiod (42 °N lat.) with day-extension lighting provided by 400 W high-pressure sodium (HPS) lamps (LU400; GE, Canada) supplying a supplemental photosynthetic photon flux (PPF) of 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant height as measured by a custom made line quantum sensor with ten photodiodes (Apogee Instruments, Inc., Logan, UT, USA). The HPS lamps were operated by an environmental control computer (Priva Integro 724, Priva, Vineland Station, Ontario). When irradiance was less than 580 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for at least ten minutes supplemental lighting was activated. Lighting would shut off only when irradiance was greater than 580 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for a minimum of 20 min. Average temperatures and standard deviations are reported in Table 2.1. Plants were irrigated as needed with reverse osmosis water supplemented with a water-soluble fertilizer supplying (mg l^{-1}): 125 N, 12 P, 100 K, 65 Ca, 12 Mg, 1.0 Fe and Cu, 0.5 Mn and Zn, 0.3 B, and 0.1 Mo (MSU RO Water Special, GreenCare Fertilizers, Inc., Kankakee, IL, USA). On 17 April 2012 (run 1) and 1

October 2012 (run 2) plants were pinched back one node and treated with 200 $mg \cdot L^{-1}$ ethephon (Florel, Southern Agricultural Insecticides, Inc., USA) diluted with reverse osmosis water. On 28 April 2012 (run 1 only) flowering was observed on genotype 10-43-41 only. Therefore, all plants of this genotype were pinched so only one node per branch remained and a second ethephon application of the same concentration was administered on 2 May 2012. At the onset of the experiment, the number of lateral branches exceeding 3 cm in length and the number of nodes per branch were recorded and the 36 most uniform cuttings per genotype were selected for the experiment. Plants were placed into their respective treatments on 3 May 2012 (run 1) and 5 October 2012 (run 2), and spaced 23 cm from mid-pot to mid-pot. After four weeks in treatment, all plants were repotted into 15-cm square plastic containers (2506 ml) filled with the same medium used before and spaced 36 cm from mid-pot to mid-pot. Plants were grown in treatment for nine weeks following the last ethephon application.

DLI treatments. Six different DLIs were created using two different woven shade cloths (OLS 50, OLS 30, Ludvig Svensson Inc. Charlotte, NC) or no shade cloth, over two time periods (May-July and October-December 2012, runs 1 & 2 respectively), resulting in average DLIs ranging from 3.55-20.31 $mol \cdot m^{-2} \cdot d^{-1}$ (Table 2.1).

Tissue sampling and steviol glycoside analysis. Following nine weeks in treatments, leaf tissue samples were collected for quantification of steviol glycosides. Ten fully expanded leaves randomly selected from about 66% of the height of the plant (from the base) were collected and dried at 65 °C for at least 72 h, then transferred to 15 mL conical tubes and held at -20 °C. Ten samples per genotype from each treatment were selected randomly for analysis. Samples were allowed to warm to room temperature before seven stainless steel ball bearings (3.97 mm diameter, 440C -G16, from VXB Ball Bearings, NationSkander California Corp., Anaheim, CA)

were added to each tube. Samples were ground via shaking with an adapted paint shaker for 15 min until the sample was a fine powder. Ten to twelve milligram samples were transferred to 1.5 mL centrifuge tubes and the mass was recorded for each sample. One milliliter of extraction buffer (final concentration 6.78 M ethanol, and 0.1mM digitoxin) was added to each sample. Samples were shaken using a vortex mixer with microtube foam insert (Scientific Industries Inc. Bohemia, NY, USA) for 10 min at room temperature. Samples were then centrifuged at 21,000 g_n for 10 min at 4 °C. The supernatant was transferred to a new 1.5 mL centrifuge tube. Extract supernatant was prepared for analysis by using a Millipore deep well filter plate and deep well 96-well receiving plate as described by Shafii et al., (2012). Samples were analyzed via ultrahigh performance liquid chromatography-tandem mass spectrometry method as described by Shafii et al., (2012) with the exception that only five steviol glycosides were separated and quantified: stevioside, and rebaudiosides A, B, C and D. The optimization parameters for rebaudioside D in the 2012 analysis are reported in Table 2.2.

Morphological data collection. At the time of tissue collection for glycoside analysis, the number of nodes on the tallest branch (opposite and alternate nodes were each counted as one), canopy height (cm, from soil line to tallest point), and average leaf area (one leaf measured twice collected from the seventh node down from the apex of the tallest branch) were determined. Leaf area was calculated by averaging two measurements of the same leaf using a leaf area meter (LI-3000; LI-COR, Lincoln, NE).

Statistical analysis. Six DLI environments were generated over the two runs. Normality of all data was checked by applying the Shapiro-Wilk statistic to the residuals of the data in PROC UNIVARIATE procedure in SAS version 9.3 (SAS Institute, Cary, N.C.). Homogeneity of variances was ensured by examining residual plots. Concentrations of stevioside and

rebaudiosides A, C and D (log transformation) and proportion of rebaudioside B (square root transformation) were transformed to meet ANOVA assumptions for normality. Analysis of variance and means separation (Tukey's $HSD_{(0.05)}$) were conducted using PROC GLM. Genotype, run, DLI, and interactions were included in the model.

Experiment 2

Plant material and propagation. On 27 March 2013 genotype 10-43-41 was vegetatively propagated into plug trays [72-cell size square (59 ml volume)] in the same soilless medium and propagation environment as described in experiment 1. On 24 April 2013, when plant roots reached all corners of the plug cell and vegetative growth was observed, rooted cuttings were transplanted into larger plug trays [50-cell size, round (76 mL volume)] with media composed of 100% Suremix Perlite potting mix.

Field environment and plant culture. On 7 June 2013, four plants were transplanted into each of 5 plots in each of three white plastic-covered north-south oriented raised rows (15 cm high by 66 cm wide), for a total of 15 plots, in a capac loam soil field at the Michigan State University Horticulture Teaching and Research Center (Holt, MI, USA). Soil pH measured was at 6.7 and the average electrical conductivity was 0.51 mS. Transplants were positioned in two staggered north- south rows per plastic-covered row, set 15 cm inside from each plastic-covered row edge. Within-row spacing was 46 cm; between-row spacing was 36 cm. Irrigation was supplied via natural rainfall as well as by drip tape laid underneath the plastic in each row. Supplemental irrigation was only utilized on four dates: 15, 17, 25 and 26 July 2013 on the basis of plant need. Pre-plant fertilizer was applied supplying (kg/ha) 79.9 N, 34.9 P, 66.3 K. The light environment was created by the natural photoperiod and daily light integral (42 °N

latitude). Minimum, maximum, and average daily temperature and daily light integral was recorded by a miniature weather station situated near the experimental treatments (Watch Dog WeatherTracker Model 305, Spectrum Technologies, Aurora, IL, USA). The average temperatures with standard deviation for each minimum, maximum, and average daily temperatures were 14.6 ± 3.8 °C, 27.30 ± 3.9 °C, 20.56 ± 3.5 °C, respectively. Temperatures ranged from 6 – 35 °C over the course of the experiment. Daily light integrals and temperature for the experimental period were recorded (Figs 2.1 and 2.2, respectively). The experiment ran for eight weeks.

DLI treatments. To create different DLI environments within the field, metal conduit frames were constructed and covered with different shade cloths (Figure 2.3). Each frame consisted of three pieces of 1.1 cm diameter metal conduit bent into an open-bottomed pentagonal shape, with additional pieces of straight conduit positioned vertically at each bend point for support. Twelve matching frames were assembled, each measuring 200.7 cm long by 121.9 cm wide, 109.2 cm from bottom to peak. Frames were secured in the field by fitting each of the six vertical frame legs over a 90 cm piece of rebar driven 30 cm into the ground. Thus, the east and west walls of the frame were 15 cm from either side of the plastic-covered row. Within each of the three rows, five different DLIs averaging 10.32 - 39.70 $mol \cdot m^{-2} \cdot d^{-1}$ were created (Table 2.3). DLI treatments were achieved by covering the frames with one of three different woven shade cloths (Ludvig Svensson Inc.) independently (OLS 60, OLS 50, OLS 30), a combination of two shade cloths to achieve a heavy shade treatment (OLS 40 plus OLS 30), or no shade cloth. Shade cloth was secured to the frames using cable ties.

The experiment was laid out in a randomized complete block design, with each of three plastic-covered rows representing a block. Spacing between treatments within a block was

equivalent to the length of one shade frame, or 200.7 cm. DLI treatments were randomized within a block.

Flowering data collection, tissue sampling and steviol glycoside analysis. Flowering data was collected at two time points during the experiment, after four and eight weeks in treatment. Data consisted of noting whether each plant was visibly vegetative, had visible flower buds, but no open flowers, or contained open flowers. Following eight weeks in treatments, tissue samples were collected from each plant individually for quantification of steviol glycosides. Collection was performed in the same manner as described for Experiment 1, except that, after drying, samples were placed into glass desiccators and stored at room temperature.

Tissue samples were transferred to 15 mL conical tubes. Samples were ground, extracted and analyzed for steviol glycoside concentration as described for experiment 1, with the exception that rebaudioside X was quantified while rebaudioside B was not (Table 2.2).

Statistical analysis. The experiment employed a completely randomized block design. Five DLIs with three replicates, each containing four experimental units were analyzed. Normality of the data was evaluated by applying the Shapiro-Wilk statistic to the residuals of the data in PROC UNIVARIATE procedure in SAS version 9.3 (SAS Institute, Cary, NC). Homogeneity of variances was confirmed by examining residual plots. After removing one outlier from the 11.11 $mol \cdot m^{-2} \cdot d^{-1}$ treatment group, data met ANOVA assumptions. Analysis of variance and means separation (Tukey's $HSD_{(0.05)}$) were conducted using PROC GLM. Replication, flowering, and DLI, and their interactions were included in the model.

Results

Experiment 1

Morphological traits. Morphological traits were differentially impacted by DLI, run, and genotype (Table 2.4). Node number was influenced by the interaction DLI × genotype × run. Node number for 10-43-41 did not differ between DLIs or runs, whereas 11-464 varied between both runs and treatments (Table 2.5). Genotype, run, and DLI impacted canopy height. Genotype 10-43-41 was always taller than 11-464 within a treatment and run, and plant heights for run 2 were taller than plants in run 1 at comparable DLIs for both genotypes. For both genotypes, plants were shorter under a DLI of 20.31 $mol \cdot m^{-2} \cdot d^{-1}$ compared to 5.67 or 3.55 $mol \cdot m^{-2} \cdot d^{-1}$ (Table 2.5). Leaf area was affected by DLI × run and DLI × genotype interactions. Leaf area for 10-43-41 was reduced in each run when comparing the highest DLIs of 20.31 and 10.50 $mol \cdot m^{-2} \cdot d^{-1}$ to the lowest DLIs of 8.53 and 3.55 $mol \cdot m^{-2} \cdot d^{-1}$, respectively. Leaf area for 11-464 was reduced under a DLI of 10.50 $mol \cdot m^{-2} \cdot d^{-1}$ compared to DLIs of 5.67 and 3.55 $mol \cdot m^{-2} \cdot d^{-1}$ (run 2 only, Table 2.5).

Steviol glycoside concentrations. Individual glycoside concentrations were differentially affected by genotype, DLI, run and interactions of those factors (Table 2.6.) Stevioside concentration was affected by the interaction DLI × run. Stevioside increased with increasing DLI in run 2, but decreased as DLI increased in run 1 (Figure 2.4A). Rebaudioside A concentration was affected by the interaction genotype × run. The genotypes differed in rebaudioside A content at similar DLIs. DLI × run also impacted rebaudioside A concentration. However, for both genotypes, rebaudioside A concentration increased as DLI increased (Figure 2.4B).

Rebaudioside B concentration was affected by a DLI × run × genotype interaction. Generally, rebaudioside B concentration increased with increasing DLI (Figure 2.4C), but the genotypic response differed in magnitude following the same DLI and runs. Rebaudioside C concentrations were also impacted by genotype × run and DLI × run interactions. Rebaudioside C concentration generally increased with increasing DLI (Figure 2.4D).

The concentration of rebaudioside D was influenced by the interactions DLI × run and genotype × run. The genotypes responded similarly during run 1, but in run 2 the genotypes responded differently to DLI (Figure 2.4E). DLI did not impact rebaudioside D concentration as a main effect.

The total steviol glycoside concentration (the total of the five glycosides assayed) was impacted by the interaction of DLI × run. The increase in total glycoside concentration with increasing DLI was more extreme in run 2 than in run 1, possibly because DLIs were lower and therefore more limiting in run 2. Total steviol glycoside concentration increased with DLI up to 11.87 and 8.53 $mol \cdot m^{-2} \cdot d^{-1}$, for genotypes 10-43-41 and 11-464 respectively (Figure 2.5).

Steviol glycoside proportions. The relative proportions of individual glycosides (percentage of individual glycoside calculated from total glycosides) were all affected by DLI (Table 2.7). The proportion of stevioside was affected by DLI, run, and genotype. The highest proportion of stevioside was associated with the lowest DLI of 3.55 $mol \cdot m^{-2} \cdot d^{-1}$, while the lowest proportion was associated with the highest DLI of 20.31 $mol \cdot m^{-2} \cdot d^{-1}$, although the proportions varied by genotype and were also affected by run (Figure 2.6).

Rebaudioside A proportion was also influenced by genotype, DLI and run. A DLI of 20.31 $mol \cdot m^{-2} \cdot d^{-1}$ resulted in rebaudioside A proportions that were higher compared to all other

treatments, although proportions varied by genotype and were higher in run 1 than run 2 (Figure 2.6).

The proportion of rebaudioside B was affected by the three-way interaction of genotype × DLI × run. The lowest proportion of rebaudioside B occurred at a DLI of 3.55 mol·m^{-2}·d^{-1}, but differed by genotype and run. Rebaudioside B proportion was lower in run 2 than run 1 (Figure 2.6).

Genotype, run and DLI impacted rebaudioside C proportion, which increased as DLI increased. A DLI of 20.31 mol·m^{-2}·d^{-1} yielded the highest proportion of rebaudioside C for both genotypes, which differed from the two lowest DLI treatments, 3.55 and 5.67 mol·m^{-2}·d^{-1}. Although the proportion of rebaudioside C differed between genotypes, both had higher proportions in run 1 compared to run 2 (Figure 2.6).

The proportion of rebaudioside D was affected by the interaction of all three main effects, DLI × genotype × run. The maximum percentage occurred at a DLI of 5.67 mol·m^{-2}·d^{-1}, which only differed from the "full light" (10.50 and 20.31 mol·m^{-2}·d^{-1}) treatments from each run. Genotype 10-43-41 had higher proportions of rebaudioside D compared to 11-464 (Fig 2.6).

Experiment 2

Steviol glycoside concentrations. Stevioside concentration was higher at 10.32 mol·m^{-2}·d^{-1}DLI than the 24.21 and 39.70 mol·m^{-2}·d^{-1} (Table 2.8). Rebaudioside A and C concentrations were affected by the interaction of rep × DLI. Concentrations of rebaudiosides A and C only were higher in rep 1 of the 10.32 mol·m^{-2}·d^{-1} DLI than the other two reps (Figure 2.7). Additionally, rebaudioside A concentration in rep 1 of the 11.11 mol·m^{-2}·d^{-1}DLI was higher than the other reps. Rebaudioside D concentration was affected by DLI, decreasing as DLI increased

above 11.11 $mol \cdot m^{-2} \cdot d^{-1}$ (Table 2.8). Rebaudioside X concentrations were not affected by any factor.

Total steviol glycosides remained relatively constant across treatments, staying within a range of 8.3 $mg \cdot g^{-1}$, or 10% of the averaged totals (Table 2.8). Total steviol glycosides were influenced by rep, so this was further investigated (Table 2.8). Rep 1 was found to differ from the other two reps with a higher mean concentration of total steviol glycosides due to the increased concentrations of rebaudioside A and C (Figure 2.7).

Steviol glycoside proportions. Average DLI affected the proportions of steviol glycosides (Table 2.9). The proportion of stevioside decreased as DLI increased to 24.2 or 39.7 $mol \cdot m^{-2} \cdot d^{-1}$, the same treatments where rebaudioside A proportion was largest. Similarly, the proportion of rebaudioside C was greatest at the highest DLI, 39.7 $mol \cdot m^{-2} \cdot d^{-1}$. The proportion of rebaudioside D decreased from 12.4% to 10.0% as DLI increased from 10.3 to 39.7 $mol \cdot m^{-2} \cdot d^{-1}$. The proportion of rebaudioside X was not affected by any of the factors in this experiment.

Discussion

Environmental parameters, specifically light, can affect steviol glycoside synthesis (Ceunen & Geuns, 2012, 2013a; Ceunen et al., 2012; Ermakov & Kochetov, 1994; Kumar et al., 2013; Metivier & Viana, 1979; Valio & Rocha, 1977; Yermakov & Kochetov, 1996; Zaidan et al., 1980). Our results show that DLI affects glycoside concentration, as well as the relative proportions of specific glycosides. Individual glycosides were differentially impacted by DLI, and interactions with other factors such as genotype, indicating that individual glycoside accumulation was affected by interactions between environmental and genetic factors. In general, the species *Stevia rebaudiana* possesses extreme genetic variability, probably due to

high rates of outcrossing resulting from self-incompatibility of the flowers (Singh et al., 2008). Also, sexually propagated plants are known to possess more variability in phenotype and glycoside content than those propagated asexually (Tamura et al., 1984). The two genotypes utilized in this study, 10-43-41 and 11-464, although derived from the same population (Morita II) were originally grown from seed. These two genotypes differed from each other in both morphological characters and glycoside content, likely caused by genetic differences.

Varying irradiance levels could affect steviol glycoside biosynthesis by influencing photosynthetic production and overall substrate availability, and/or differential enzyme activity by means of impacting photosynthesis. In a previous study, stevia plants were germinated and grown on media supplemented with varying amounts of sucrose. As media sucrose concentration increased from 3% to 5%, total steviol glycoside concentration increased over four-fold (Guleria et al., 2011), which the authors attributed to an enhancement of the transcriptional triggers, or cues which mediate transcription of the genes, responsible for steviol glycoside accumulation. In this study, an increase in total steviol glycosides was observed as DLI increased to ca. 8 $mol \cdot m^{-2} \cdot d^{-1}$, above which total steviol glycoside concentration was similar. This response could suggest a relationship where reducing primary productivity under lower DLIs restricts the plant's potential to synthesize secondary metabolites.

Guleria et al. (2011) also found that expression of UGT85C2, the gene encoding the enzyme that glycosylates steviol to steviolmonoside (Richman et al., 2005a) which is the first major step that occurs in the cytosol in the steviol glycoside biosynthetic pathway, increased in plants exposed to 5% sucrose in media compared to those with 1% or 3% sucrose exposure. In addition to UGT85C2, Guleria et al. (2011) discovered that other pathway specific genes CDPS, KO, and KS, which function upstream of glycosylation events in the steviol glycoside

biosynthetic pathway, also had higher expression in 5% sucrose treatments. If expression of these upstream genes is stimulated by exogenous application of sucrose, it is possible that expression of steviol glycoside biosynthetic enzymes could also be affected by differences in photosynthetic rates in response to varying irradiance.

The availability of sucrose may indirectly influence the production and/or accumulation of certain glycosides by increasing expression of UGT76G1. UGT76G1, which is known to be involved in the glycosylation of stevioside to rebaudioside A, dulcoside A to rebaudioside C and steviolbioside to rebaudioside B, exhibited increased expression in plants exposed to 5% sucrose, compared to the 1% and 3% treatments (Guleria et al., 2011). These three analytes, which were quantified in experiment 1 (rebaudiosides A, B and C) and experiment 2 (rebaudiosides A and C only), all increased in both concentration and proportion (Tables 2.8 and 2.9; Figs. 2.4 and 2.6) as DLI increased. Although total steviol glycosides increased with increasing DLI to a certain point in experiment 1, not all glycosides followed the same pattern. In experiment 1, rebaudioside A, B and C concentrations increased as DLI increased, however rebaudioside D did not (Figure 2.4). Since rebaudioside A is believed to be the major precursor for rebaudioside D, it appears that rebaudioside D synthesis is not being limited by precursor availability.

The glycosylation of rebaudioside A to rebaudioside D is believed to be mediated by another enzyme, UGT91D2 (Kishore et al., 2011). Both stevioside and rebaudioside D decreased in proportion as DLI increased in experiment 2 (Table 2.9; Figure 2.6). Although UGT91D2 was not examined in the study by Guleria et al. (2011), UGT74G1 was included. One of the functions of UGT74G1 is to glycosylate steviolbioside to form stevioside (Richman et al., 2005a). The study showed that 3% sucrose-treated plants yielded higher relative expression of UGT74G1 than did those plants supplied with 5% sucrose. If expression of UGT74G1 is

stimulated by a moderate level of sucrose, this could partly explain why stevioside concentration decreased as DLI increased in experiment 2. It is possible that expression of UGT91D2 is also increased when moderate or low levels of sucrose are available. The results of experiments 1 and 2 show that glycosides may have different DLI optima for maximum biosynthesis, and should be examined on an individual basis when referring to DLI effects. Individual glycosides could be grouped based on the UGTs responsible for their biosynthesis.

Experiments 1 and 2 were conducted in a greenhouse and field setting, respectively, in order to achieve a wide range of DLIs. Because these environments could not be identical, other factors aside from the DLI may have played a role in the variation observed in the results of these experiments. Brandle and Rosa (1992) stated that glycoside levels in leaf tissue can be affected by environment, specifically agronomic practices, which Shock (1982) specified as fertilizer inputs and harvest regimes. The effects of day length have also been examined and found to be important for glycoside accumulation (Metivier & Viana, 1979) as well as the spatio-temporal allocation of those glycosides (Ceunen & Geuns, 2013a).

One environmental difference that was not accounted for during the two runs of experiment 1 is the contrast in light quality due to differing amounts of time that supplemental lighting was engaged. High-pressure sodium lamps deliver a higher ratio of red to far-red light than natural sunlight (Ballare & Scopel, 1994). Different red to far-red ratios influence phytochrome photoequilibria and phytochrome signaling (Franklin & Quail, 2010) resulting in differential gene expression. One study examining the effects of red light on steviol glycoside biosynthesis found that plants exposed to one hour of night-interruption red light produced higher amounts of total steviol glycosides, although the proportions of measured glycosides remained relatively constant (Ceunen et al., 2012). The authors hypothesized that the

phytochrome-mediated responses have an effect on the upstream genes in the steviol glycoside pathway, and not the more downstream UGTs responsible for the glycosylation of steviol (Ceunen et al., 2012). In this study (experiment 1), fairly comparable DLIs were created in run 1 and run 2, averaging at 11.87 and 10.50 $mol \cdot m^{-2} \cdot d^{-1}$, respectively. The fall months during which run 2 was conducted supplied much less natural light than the summer months of run 1. Therefore the supplemental lighting would have been used more often in run 2, especially in the evening and morning hours since day length is also shorter. Stevioside concentrations were considerably higher in run 2 (18.07 mg/g), compared to run 1 (10.88 mg/g). Perhaps phytochrome-mediated responses can affect the steviol biosynthesis pathway, or particular components such as stevioside.

Plants were grown under long days in both experiments, although the day length was not identical (16 h in experiment 1, range of ca. 15.3 -14.3 h in experiment 2). This difference could account for some of the variability in stevioside concentration between experiments since UGT74G1 has also been found to have significantly lower expression levels in upper leaves under 16 h days compared to 12 h days (Mohamed et al., 2011). Although day length has been shown to differentially impact the expression of UGTs 74G1, 76G2 and 85C2, the differences in day lengths between experiments 1 and 2 were not substantial.

The difference in soil water status between experiments (expts.) 1 and 2 may have contributed more to variation in stevioside concentration than temperature differences between the environments. A previous study conducted in a field setting found temperature did not influence stevioside production, although stevioside production was affected by rainfall (Nepovim et al., 1998). Stevioside concentration varied more between expt. 1 and expt. 2 than between runs 1 and 2 during expt 1. Experiment 1 was conducted in a greenhouse, and water

availability should not have been a limiting factor, since it was consistently applied throughout both runs. Experiment 2 provided a different soil environment than experiment 1, and likely had greater fluctuations in soil water status, despite the use of irrigation.

Perhaps one of the largest differences between the growing conditions in expt 1 versus expt 2 is the fluctuation in day and night temperature. Because experiment 1 was conducted in a greenhouse, temperature remained fairly constant over the course of the experiment, and overall, run 1 averaged about 4 °C warmer than run 2 (Table 2.1). Since experiment 2 was performed outdoors, the plants were subjected to a wider range of temperatures (Table 2.8), and specifically a positive difference between light and dark period temperatures (DIF). DIF has been proven to influence secondary metabolite concentration, specifically a greater positive DIF increased anthocyanin content in *Perilla frutescens* var. *acuta* (Park et al., 2013).

This study has shown that, although the general response by stevia genotypes to DLI may be similar, the degree that of response can vary by genotype. Previous studies have shown that different stevia accessions show a high level of polymorphism among 179 DNA fragments amplified (Heikal et al., 2008). The high degree of genetic variability present in stevia necessitates that more genotypes or populations be evaluated in order to apply these results to the species in general.

APPENDIX

Table 2.1. Average daily light integrals (DLI) with standard deviations, DLI minimum and maximum (24-hour average) for each treatment and average temperature (°C) with standard deviation for each DLI treatment created with shade cloth over two time periods in Michigan State University greenhouses during 2012.

Date / Treatment	DLI ($mol \cdot m^{-2} \cdot d^{-1}$) AVG ± SD	DLI (24-hr AVG) Min – Max	Temperature (°C) AVG ± SD
5 Oct – 7 Dec			
Low Light	3.55 ± 1.15	1.61 – 6.26	19.88 ± 1.30
Medium Light	5.67 ± 1.39	3.12 – 9.03	20. 57 ± 1.58
High Light	10.50 ± 2.34	6.23 – 17.96	20.38 ± 1.38
3 May – 5 July			
Low Light	8.53 ± 2.18	3.03 – 12.22	23.21 ± 3.23
Medium Light	11.87 ± 2.65	5.19 – 15.72	24.45 ± 4.01
High Light	20.31 ± 4.51	10.55 – 29.23	24.06 ± 3.66

Table 2.2. Multiple reaction monitoring (MRM) transitions and optimized instrumental parameters used for LC-MS-MS measurements of steviol glycosides.

Compound	MRM Transition	Rt	DP	EP	CE
Stevioside	803 > 641	3.34	- 45	- 7	- 40
Rebaudioside A	965 > 803	3.32	- 70	- 10	- 65
Rebaudioside C	949 > 787	3.55	- 60	- 10	- 70
Rebaudioside D	1127 > 803	2.64	- 35	- 10	- 45
Rebaudioside X	1289 > 803	2.79	- 45	- 4	- 60
Digitoxin (IS)	763 > 633	5.36	- 50	- 10	- 50

Rt retention time (in minutes), *DP* declustering potential (in volts), *EP* entrance potential (in volts), *CE* collision potential (in volts), *IS* internal standard.

Table 2.3. Average daily light integrals (DLI) with standard deviations, DLI minimum and maximum (24 hour mean) for each treatment created with shade cloth in a Michigan field during 7 June – 8 August 2013.

Treatment (% of full light)	DLI ($mol \cdot m^{-2} \cdot d^{-1}$) mean ± SD	DLI (24-hr mean) Min – Max
26%	10.32 ± 3.03	2.01 – 14.78
28%	11.11 ± 3.26	2.16 – 15.91
43%	17.07 ± 5.01	3.32 – 24.44
61 %	24.21 ± 7.11	4.71 - 34.67
100%	39.70 ± 11.66	7.72 – 56.83

Table 2.4. Summary analysis of variance showing significance of main effects and interactions for each of the morphological dependent variables from Experiment 1, canopy height, leaf area and node number. Means are based on ten plants per average DLI.

	Canopy Height (cm)	Leaf Area (cm^2)	Node Number
Source		Significance	
Genotype	***	***	***
Run	***	NS	NS
DLI	***	***	NS
Genotype * Run	NS	NS	NS
DLI * Genotype	NS	*	NS
DLI * Run	NS	***	NS
DLI * Genotype * Run	NS	NS	*

NS, *, and *** indicate non-significance or significance at $P < 0.05$ or 0.001, respectively.

Table 2.5. Means for canopy height (cm), node number (count) and leaf area (cm^2) for stevia plants exposed to six different daily light integrals (DLI).

	Run	DLI ($mol \cdot m^{-2} \cdot d^{-1}$) AVG ± SD	Canopy Height (cm)	Node Number	Leaf Area (cm^2)
10-43-41	Run 1				
		20.31 ± 4.51	52.80 a	19.8 a	9.85 a
		11.87 ± 2.65	58.00 a	20.0 a	12.54 ab
		8.53 ± 2.18	59.20 ab	19.5 a	14.21 bc
	Run 2				
		10.50 ± 2.34	66.40 bc	20.2 a	10.15 a
		5.67 ± 1.39	70.84 c	20.1 a	12.98 ab
		3.55 ± 1.15	74.14 c	20.8 a	17.01 c
11-464	Run 1				
		20.31 ± 4.51	44.70 a	23.0 a	6.45 bc
		11.87 ± 2.65	45.90 a	26.1 ab	6.85 c
		8.53 ± 2.18	46.10 a	23.2 a	7.17 c
	Run 2				
		10.50 ± 2.34	51.72 b	34.7 c	4.41 a
		5.67 ± 1.39	53.19 b	31.3 c	5.48 b
		3.55 ± 1.15	55.28 b	30.3 bc	6.17 bc

Column means were analyzed by genotype, different letters indicate significant differences across DLI treatment within a genotype as determined by Tukey's $HSD_{(0.05)}$. Samples collected following eight weeks in treatments. Means are based on ten plants per genotype.

Table 2.6. Summary analysis of variance of main effects and interactions for each of the concentration dependent variables, stevioside, rebaudiosides A – D and total steviol glycosides.

	Stevioside (mg/g)	Reb A (mg/g)	Reb B (mg/g)	Reb C (mg/g)	Reb D (mg/g)	Total (mg/g)
Source			Significance			
Genotype	NS	***	NS	***	***	NS
Run	*	***	***	***	***	***
DLI	NS	***	***	***	NS	***
Genotype * Run	NS	*	*	*	*	NS
DLI * Genotype	NS	NS	*	NS	NS	NS
DLI * Run	***	***	***	***	*	***
DLI * Run * Genotype	NS	NS	***	NS	NS	NS

NS, *, and *** indicate non-significance or significance at $P < 0.05$ or 0.001, respectively. Samples collected following nine weeks in treatment. Means are based on ten plants per average DLI.

Table 2.7. Summary analysis of variance of main effects and interactions for each of the dependent variables (the percentage of each glycoside calculated from total steviol glycosides (TSG)), stevioside and rebaudiosides A – D.

	Stevioside (percentage of TSG)	Reb A (percentage of TSG)	Reb B (percentage of TSG)	Reb C (percentage of TSG)	Reb D (percentage of TSG)
Source			Significance		
Genotype	*	***	NS	*	***
Run	***	*	***	*	NS
DLI	***	***	***	*	***
Genotype * Run	NS	NS	*	NS	***
DLI * Genotype	NS	NS	*	NS	*
DLI * Run	NS	NS	***	NS	NS
DLI * Run * Genotype	NS	NS	***	NS	*

NS, *, and *** indicate non-significance or significance at $P < 0.05$ or 0.001, respectively. Samples collected following nine weeks in treatment. Means are based on ten plants per average DLI.

Table 2.8. Mean concentrations (mg/g) of stevioside, rebaudioside A, rebaudioside C, rebaudioside D, rebaudioside X and total glycosides for stevia plants exposed to five different daily light integrals (DLI).

DLI ($mol \cdot m^{-2} \cdot d^{-1}$) AVG ± SD	Stevioside (mg/g)	Reb A (mg/g)	Reb C (mg/g)	Reb D (mg/g)	Reb X (mg/g)	Total (mg/g)
10.32 ± 3.03	32.75 b	29.72 a	5.09 a	9.77 c	1.60 a	78.93 a
11.11 ± 3.26	32.74 ab	31.35 a	5.17 a	10.10 c	1.65 a	84.06 a
17.07 ± 5.01	32.43 ab	29.53 a	5.14 a	9.34 bc	1.82 a	78.25 a
24.21 ± 7.11	30.31 a	33.35 a	5.40 a	8.00 ab	1.59 a	78.65 a
39.70 ± 11.66	28.64 a	32.32 a	5.57 a	7.63 a	1.62 a	75.80 a
Source			Significance			
AVG DLI	*	NS	NS	***	NS	NS
Rep	NS	*	*	NS	NS	*
Flowering	NS	NS	NS	NS	NS	NS
Rep * Flowering	NS	NS	NS	NS	NS	NS
AVG DLI * Rep	NS	*	*	NS	NS	NS
AVG DLI* Flowering	NS	NS	NS	NS	NS	NS
AVG DLI * Rep * Flowering	NS	NS	NS	NS	NS	NS

*Values followed by different letters indicate significant differences across DLI treatments as determined by Tukey's $HSD_{(0.05)}$. Means are based on three replicates of four plants each. NS, *, and *** indicate non-significance or significance at $P < 0.05$ or 0.001, respectively. Samples collected following eight weeks in treatment.

Table 2.9. Mean proportions (percentage of total glycosides) for stevioside, rebaudioside A, rebaudioside C, rebaudioside D, rebaudioside X and total glycosides for stevia plants exposed to five different daily light integrals (DLI).

DLI ($mol \cdot m^{-2} \cdot d^{-1}$) AVG ± SD	Stevioside (percentage of TSG)	Reb A (percentage of TSG)	Reb C (percentage of TSG)	Reb D (percentage of TSG)	Reb X (percentage of TSG)
10.32 ± 3.03	41.55 b	37. 57 a	6.44 ab	12.42 b	2.02 a
11.11 ± 3.26	42.37 b	37.32 a	6.18 a	12.15 b	1.97 a
17.07 ± 5.01	41.40 b	37.76 a	6.56 bc	11.96 b	2.32 a
24.21 ± 7.11	38.55 a	42.37 b	6.88 c	10.19 a	2.02 a
39.70 ± 11.66	37.71 a	42.80 b	7.37 d	9.97 a	2.15 a
Source			Significance		
AVG DLI	***	***	***	***	NS
Rep	NS	NS	NS	NS	NS
Flowering	NS	NS	NS	NS	NS
Rep * Flowering	NS	NS	NS	NS	NS
AVG DLI * Rep	NS	NS	NS	NS	NS
AVG DLI* Flowering	NS	NS	NS	NS	NS
AVG DLI * Rep * Flowering	NS	NS	NS	NS	NS

*Values followed by different letters indicate significant differences across DLI treatments as determined by Tukey's $HSD_{(0.05)}$. Means are based on three replicates of four plants each. NS, *, and *** indicate non-significance or significance at $P < 0.05$ or 0.001, respectively. Samples collected following eight weeks in treatment.

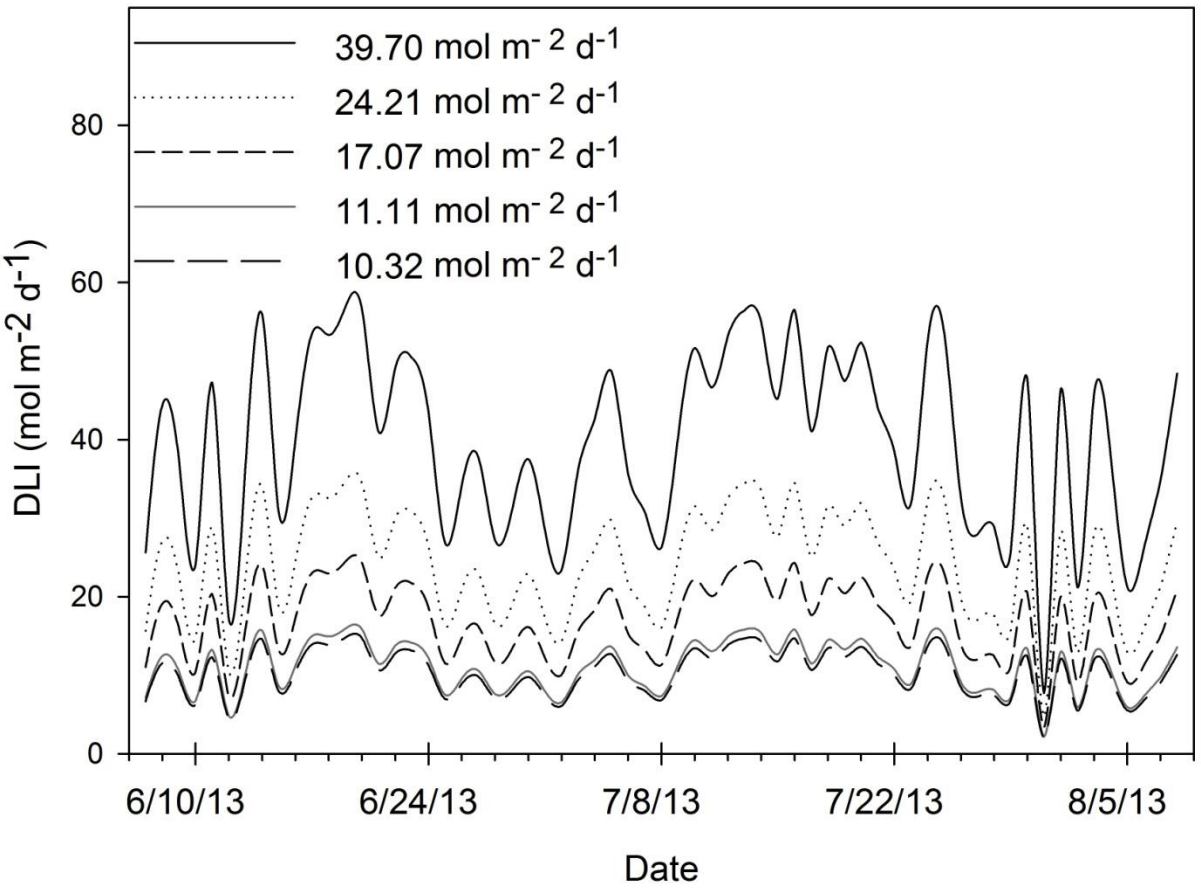

Figure 2.1. Daily light integrals (DLIs) for treatments consisting of full sunlight and four levels of shading from 7 June – 8 August 2013 (Experiment 2).

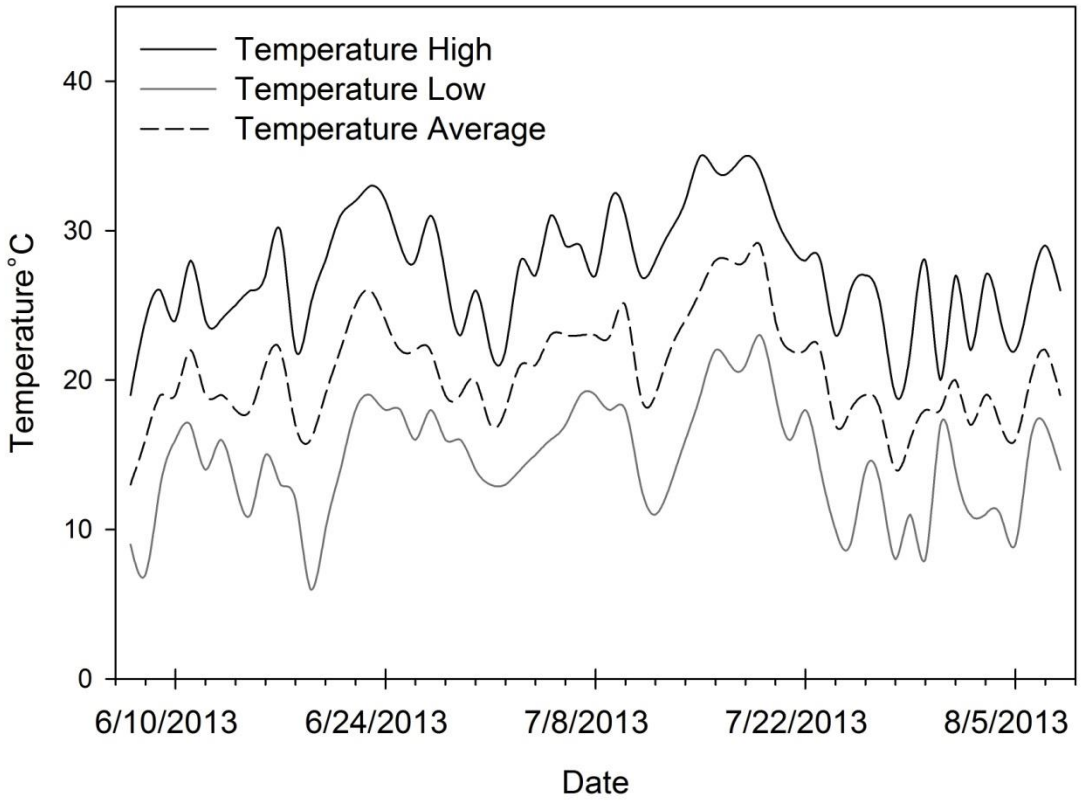

Figure 2.2. Temperature data for all DLI treatments from 7 June – 8 August 2013 (Experiment 2).

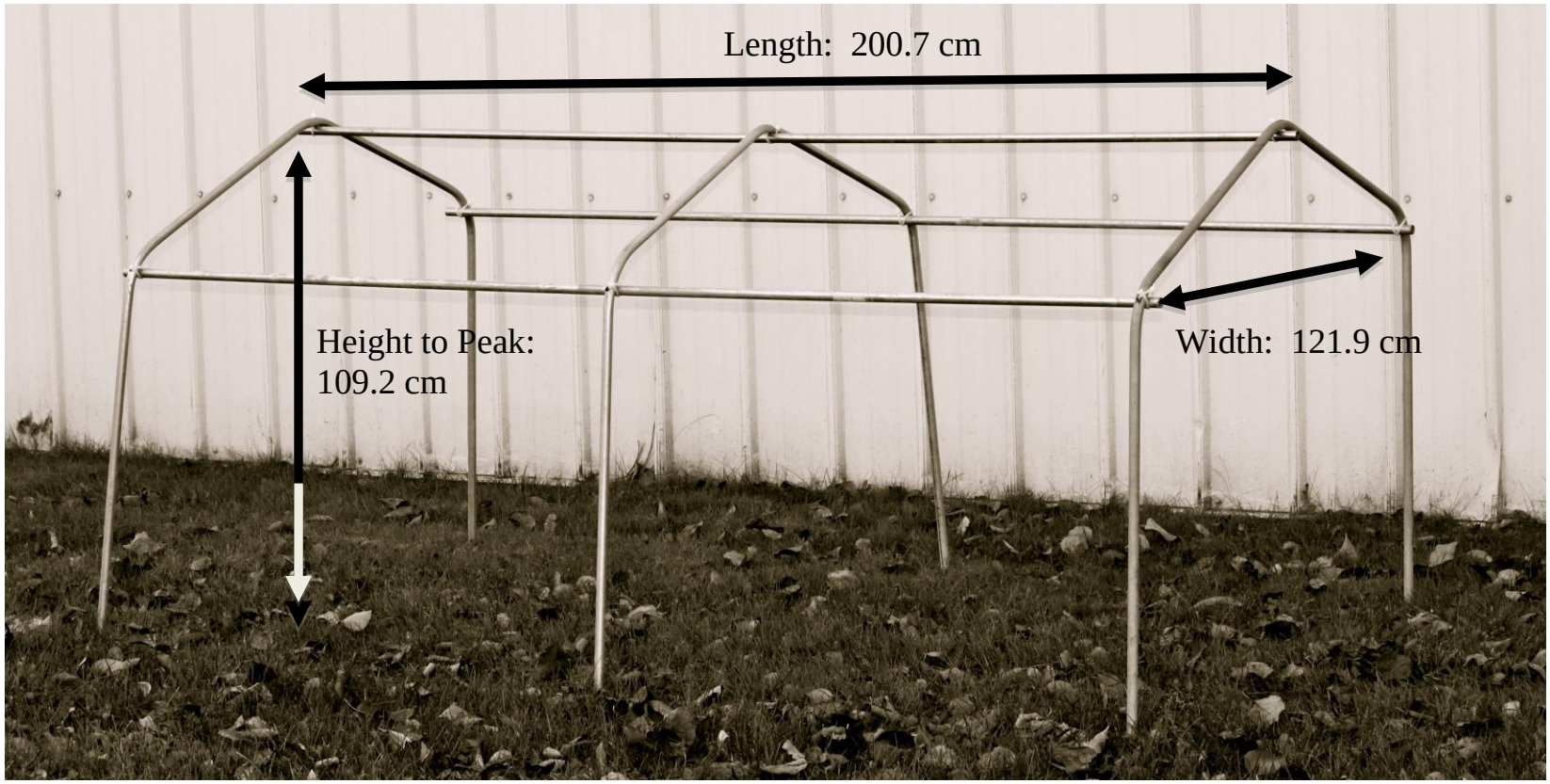

Figure 2.3. Frame dimensions for one of twelve identical frames constructed for use in Experiment 2.

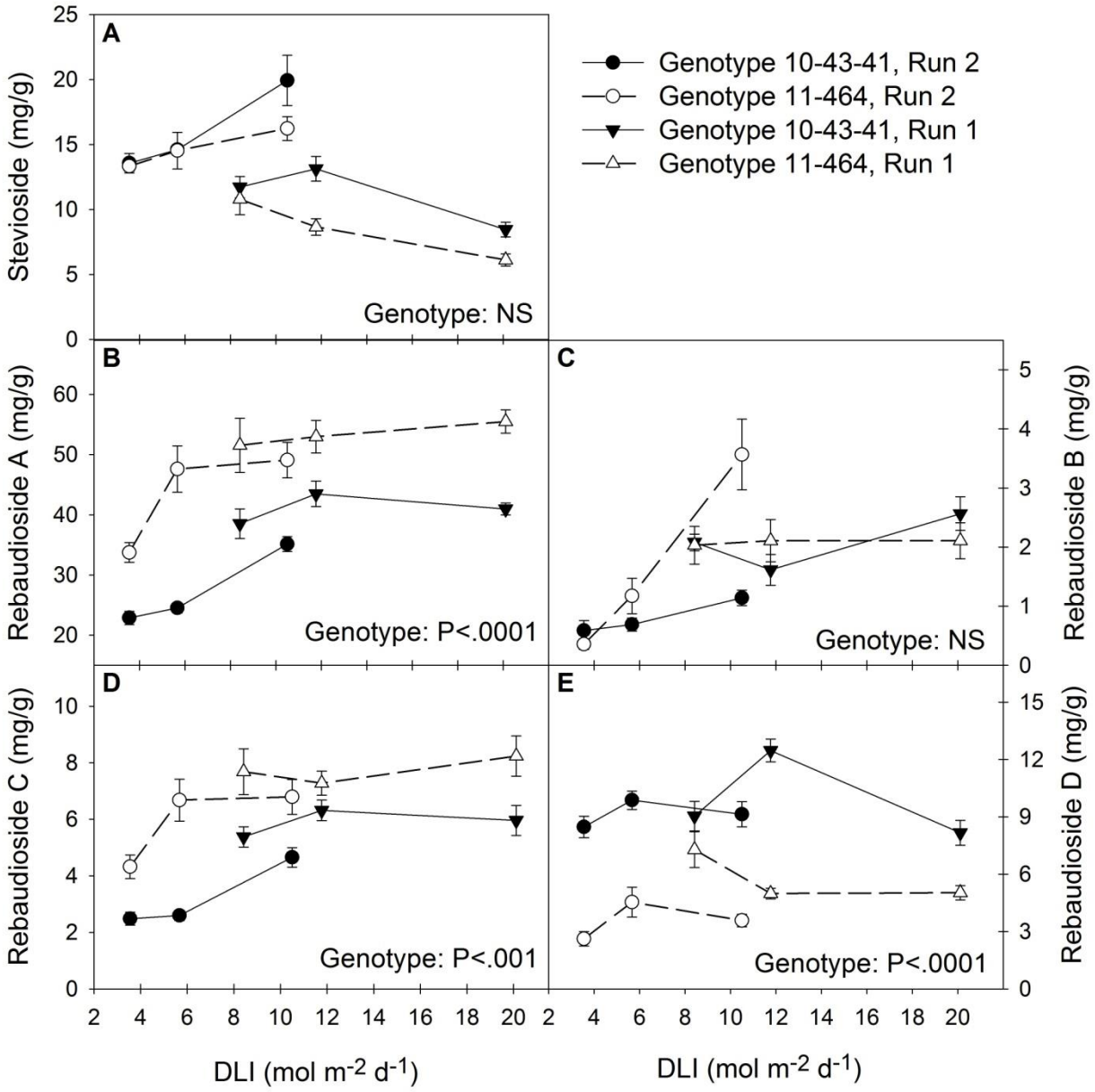

Figure 2.4. Individual steviol glycoside concentration (mg/g) versus average DLI, including stevioside (A), reb A (B), reb B (C), reb C (D), and reb D (E). Genotypes 10-43-41 and 11-464 are represented by filled and open symbols; runs 1 and 2 are distinguished by triangles and circles, respectively.

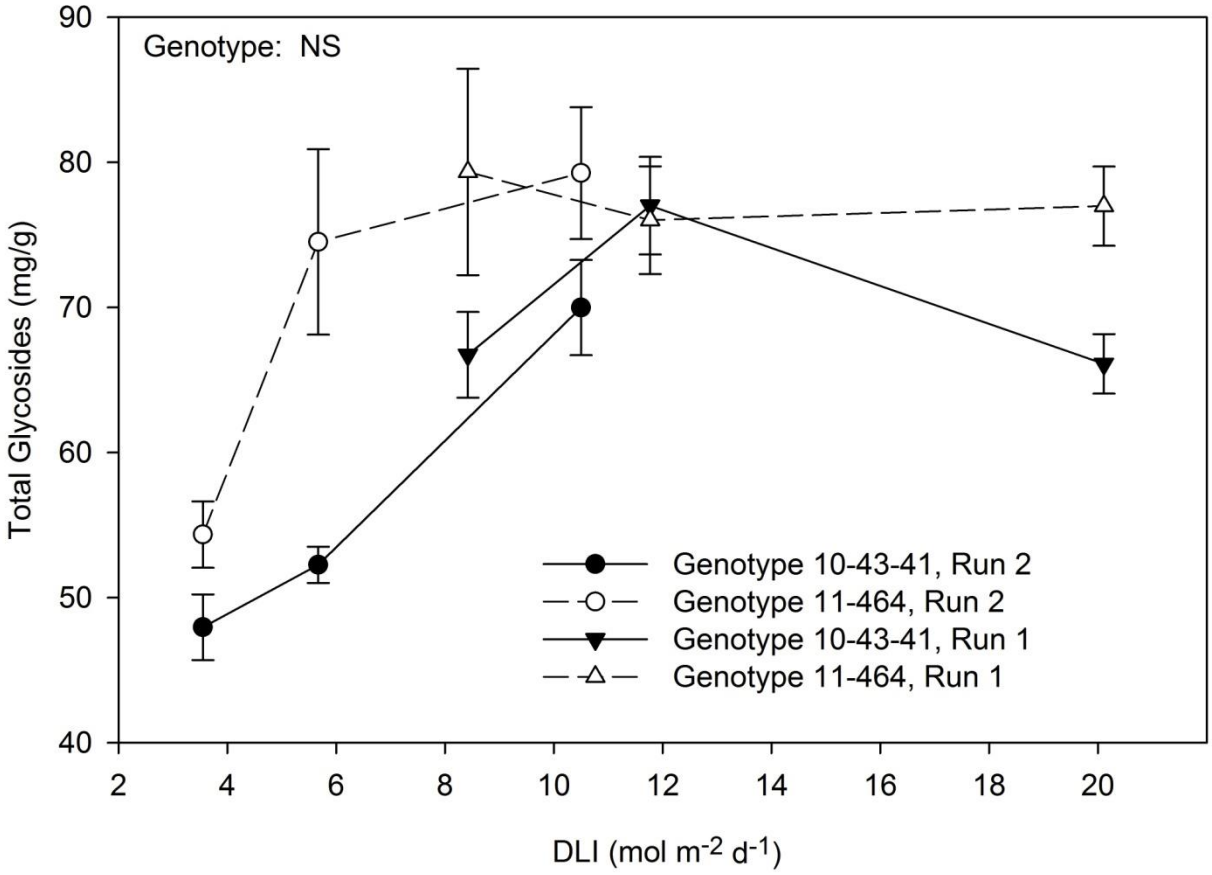

Figure 2.5. Total steviol glycoside concentration (mg/g) versus average DLI ($mol \cdot m^{-2} \cdot d^{-1}$). Genotypes 10-43-41 and 11-464 are represented by filled and open symbols; runs 1 and 2 are distinguished by triangles and circles, respectively.

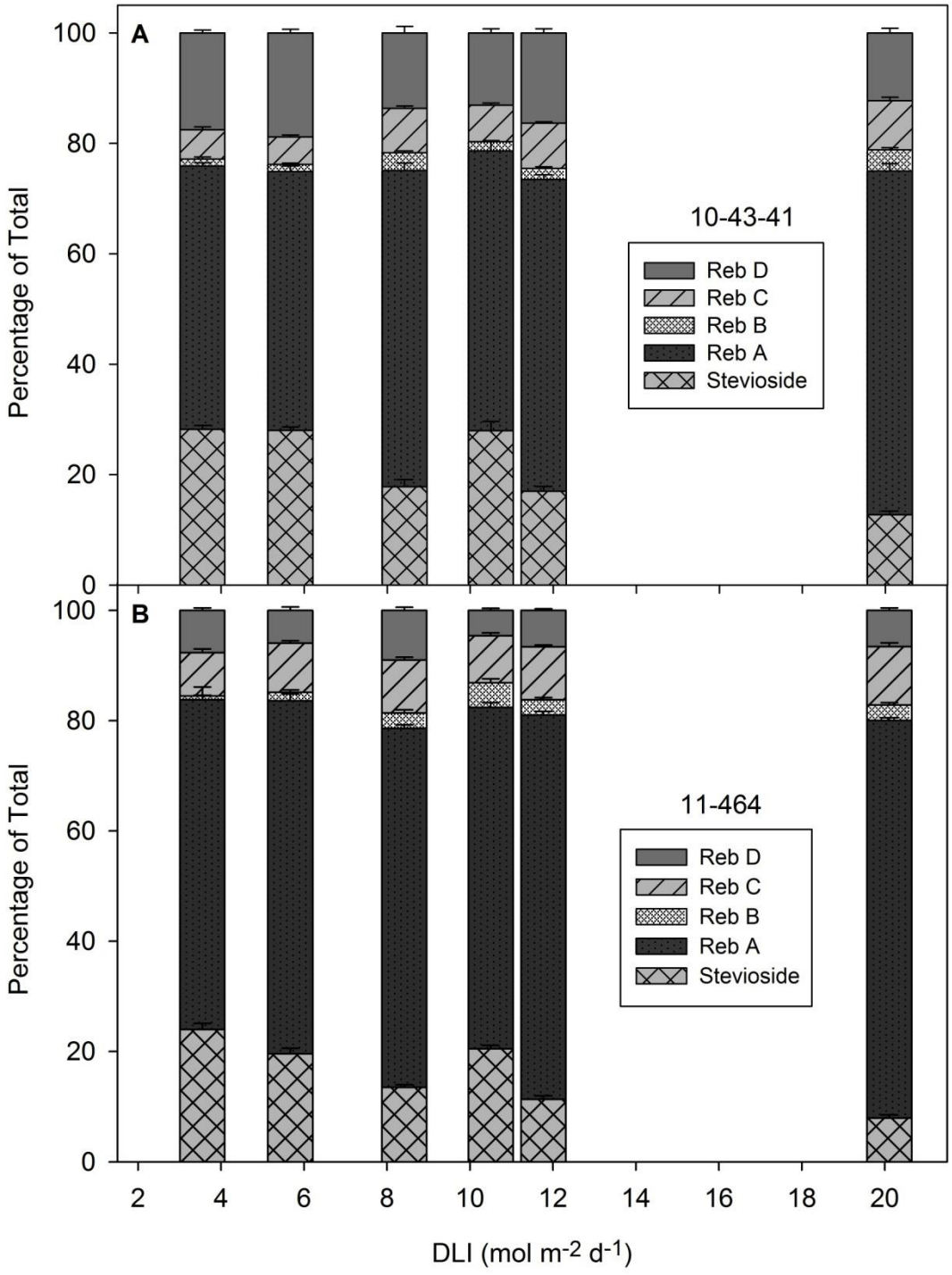

Figure 2.6. Mean proportions of stevioside and rebaudiosides A-D for each of the six average DLIs created in experiment 1 for genotypes (A) 10-43-41 and (B) 11-464.

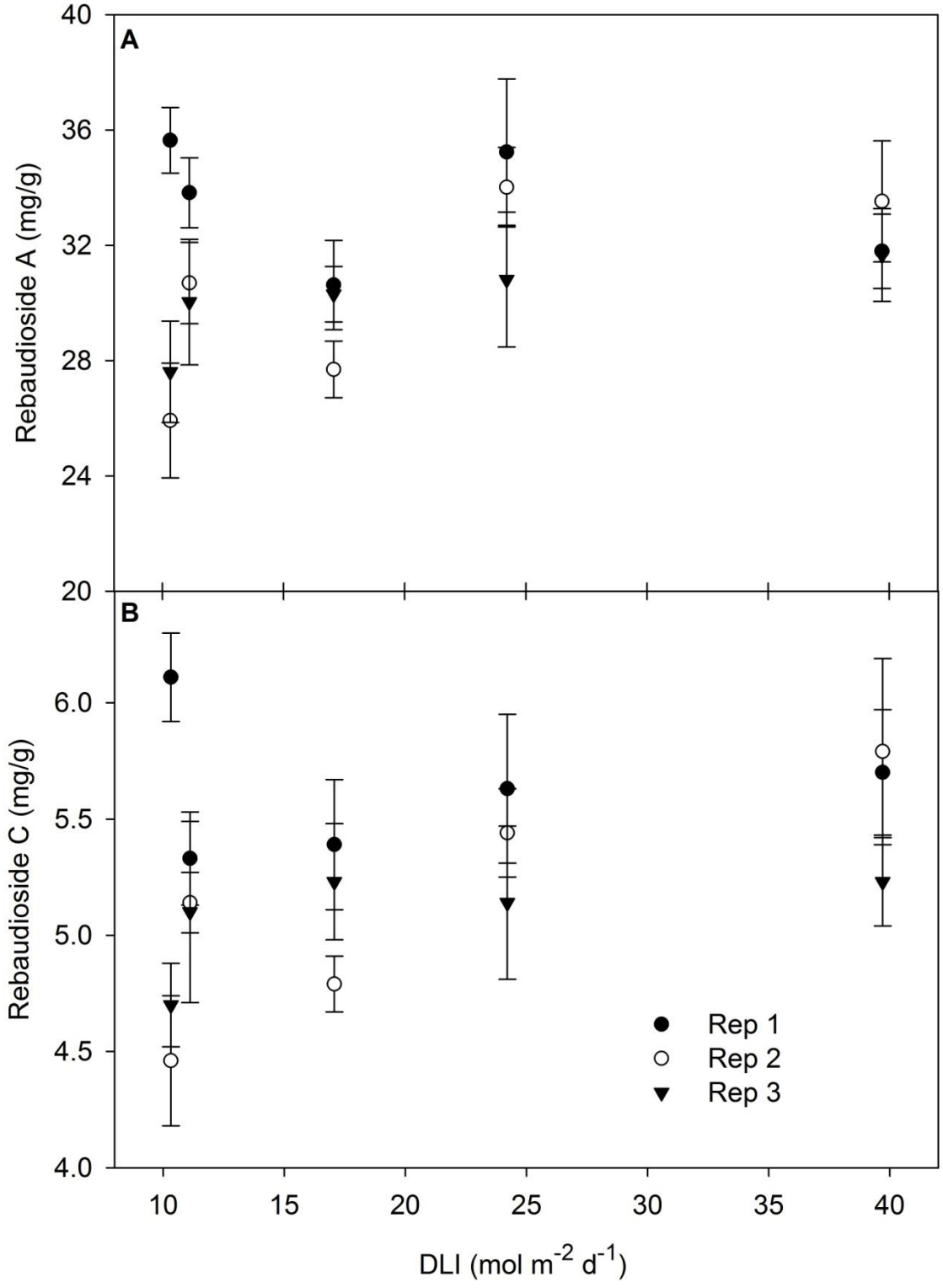

Figure 2.7. Mean concentrations of rebaudioside A (A) and rebaudioside C (B) with standard errors for each replicate in each DLI treatment after eight weeks (Experiment 2).

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LITERATURE CITED

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CHAPTER THREE

THE GENETIC CONTROL OF REBAUDIOSIDE D SYNTHESIS

Introduction

Stevia rebaudiana is gaining popularity as an alternative to caloric sweeteners, and therefore gaining momentum as a crop of economic importance. The global stevia market was valued at $304 million in 2013, with worldwide sales estimated at 4,100 metric tons of dried leaf material (Zenith International, 2013). These numbers represent a 6.5% increase from 2012, a gain that is fueled by a consumer demand for a natural, zero-calorie sweetener.

Steviol glycosides are formed by a series of glycosylation reactions that transfer activated sugars to receptor molecules, and the pattern of these glycosylations can heavily influence the taste perception of each compound (Richman et al., 2005). Rebaudioside A has been utilized in stevia-sweetened products such as diet soft drinks, and has been reported to have a bitter off-taste. When these same products are sweetened with rebaudioside D, they exhibit an improved taste profile with less bitterness (Lee et al., 2013). Rebaudioside X is reported to have a clean, sweet taste when compared to both rebaudiosides A and D with a sweetness profile similar to sucrose (Prakash et al., 2013). Commercial interest in both rebaudiosides D and X has risen due to their superior taste compared to rebaudioside A, which is currently found in many stevia-sweetened products.

Steviol glycoside synthesis begins with the synthesis of steviol, formed by the hydroxylation of kaurenoic acid by kaurenoic acid 13-hydroxylase (KAH) (Brandle et al., 2007). At this step, steviol is exported to the cytosol, and glycosylation reactions begin, mediated by UDP-glycosyltransferases (UGTs). Several UGTs have been identified to be involved in steviol glycoside biosynthesis. UGT85C2 is associated with the conversion of steviol to steviolmonoside (Shibata et al., 1991) by addition of a hexose sugar to the C_{13} position of steviol. UGT76G1 is involved in the synthesis of rebaudioside A from stevioside (Richman et al., 2005)

and possibly the synthesis of rebaudioside C from dulcoside A (Brandle, 1999) both through the C_{13} addition of a hexose sugar to the precursor molecule. UGT74G1 catalyzes the addition of a hexose sugar to the C_{19} position, creating stevioside from steviolbioside, rebaudioside E from stevioside, rubusoside from steviolmonoside, and rebaudioside A from rebaudioside B (Mohamed et al., 2011). Little information exists on the genetic control of more downstream steviol glycosides, including rebaudioside D and rebaudioside X. However, glycosylation of rebaudioside A to form rebaudioside D has been demonstrated *in vitro* to be mediated by the enzyme UGT91D2 via addition of a hexose sugar on the C_{19} position of rebaudioside A (Kishore et al., 2011).

Traditional breeding has been utilized to improve stevia varieties, however there are some obstacles that hinder traditional breeding efforts. Genetic variability exists in stevia (Brandle & Rosa, 1992), which is useful as a resource for trait improvement. However, the high heterozygosity is largely due to apparent self-incompatibility, preventing the development of inbred lines and F_1 hybrids (Abdullateef & Osman, 2011). Additionally, plants produce only a single seed per flower.

Traditional breeding techniques have also been utilized to investigate glycoside synthesis and relationships between glycoside synthesis and other traits of economic importance, specifically for the glycosides stevioside and rebaudiosides A and C (Brandle & Rosa, 1992; Brandle, 1999). The utilization of traditional breeding to examine rebaudiosides D and X and their relationships with other traits has yet to be investigated. These compounds are generally produced in much lower concentrations compared to stevioside and rebaudioside A, yet may be of higher economic importance due to their preferred flavor profile.

The objectives of this work were to understand the genetics of rebaudioside D synthesis, and potential genetic linkage between rebaudioside D synthesis and other glycoside and morphological traits of economic importance.

Materials and Methods

Selection of parental lines and propagation. During summer of 2012, 17 *Stevia rebaudiana* selections were identified as potential parents based on previous analysis of their steviol glycoside profile, which was sampled and evaluated as described in Chapter 2, expt. 1. The selections are from an open pollinated population derived from 'Morita II'. On 7 July 2012, at least 25 cuttings were vegetatively propagated from each potential parent into plug trays [72-cell size square (59 mL volume)] in a soilless medium consisting of 50% fine perlite (Perlite Vermiculite Packaging Industries, Inc. North Bloomfield, OH, USA) and 50% potting mix composed of peat, perlite, lime and starter fertilizer (Suremix Perlite, Michigan Grower Products Inc., Galesburg, MI USA). The cuttings were rooted in a glass-covered greenhouse, under the same conditions as specified in Chapter 2, expt. 1, with the exception that no fertilizer was supplied by the mist regime. Instead, fertilizer was applied once per week providing (mg L^{-1}): 600 N, 96 P, 504 K, 264 Ca, 144 Mg, 12 Fe and Cu, 6 Mn and Zn, 3.6 B, and 1.2 Mo (MSU Prop Special, GreenCare Fertilizers, Inc., Kankakee, IL, USA). On 14 August 2012, plants were moved to a glass-covered greenhouse.

Greenhouse environment and parental line culture. The greenhouse environment to which the plants were transferred provided the same photoperiod of 16 h (0600 to 2200 HR), supplemental irradiance (photosynthetic photon flux of 90 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at plant height), fertilization and irrigation as specified in Chapter 1, Experiment 1. The 24-h average temperature

was 20.5 °C with a standard deviation of 0.28 °C. On 6 September 2012, all plants were repotted into 15-cm square plastic containers (2506 mL) filled with 100% potting mix. On 5 October 2012 plants were pinched back one node and treated with 200 mg L^{-1} ethephon (Florel, Southern Agricultural Insecticides, Inc., USA) diluted with reverse-osmosis water. On 22 October 2012 all remaining flower buds were removed and a second application of ethephon was applied 23 October 2012, at a rate of 400 mg L^{-1}.

Selection of parental lines. On 12 November 2012, propagules from the 17 prospective parent lines were counted and individuals were visually assessed for plant health. Any plants appearing weak or diseased were culled. Those lines with at least 25 healthy individuals were deemed prospective parents. Parent lines were selected by comparing steviol glycoside profiles and selecting those which were most similar in percentages of stevioside and rebaudiosides A, B, C and D, but varying in the percentage of rebaudioside D (calculated as a proportion of rebaudioside D: rebaudioside A + rebaudioside D) in order to examine the genetic control of this portion of the pathway specifically. The selected parents were assigned into one of five categories, based on proportion of rebaudioside D: rebaudioside A + rebaudioside D): High, High-Medium, Medium, Medium-Low, and Low (Table 3.1). In total 16 crosses, utilizing eight individual lines, were designed to examine the synthesis of rebaudioside D from rebaudioside A (Table 3.2).

Crossing procedure. Pollen collection began on 26 November 2012 and continued through 1 February 2013. Every 2-3 days, or as needed, pollen was collected from pollen parents by lightly tapping inflorescences with a paintbrush. Pollen was allowed to dehisce into a clean Petri dish, then transferred to a 15 mL conical vial and held at 4 °C until use. Genotype

10-24 did not produce enough pollen to make the necessary crosses, therefore genotype 11-547, another "low" category designated genotype, was substituted.

Five mother plants were utilized per cross. On 3-4 January 2013, all open flowers and/or seed were removed from each mother plant, and all mothers were transferred a separate glass-covered greenhouse with the same light environment as described previously, and 24-h average temperature of 20.3 °C with standard deviation of 0.94 °C. The mother plants were grouped by genotype as much as possible in order to reduce the risk of pollen contamination. Once a single flower opened, the branch supporting the inflorescence was tagged, which indicated that all flowers above the tag would be pollinated. At least five inflorescences were tagged per mother plant. Untagged inflorescences were removed.

Pollinations were performed by hand, using a small paintbrush that was thoroughly washed with isopropyl alcohol and allowed to dry between uses. Nitrile gloves were worn and sprayed with isopropanol before handling each plant. Each inflorescence was pollinated at least five times. Following each pollination, respective tags were marked and dated to ensure all inflorescences were adequately and equally pollinated. Pollinations began on 7 January 2013 and concluded on 1 February 2013.

Seed collection and sowing. Mature stevia seeds naturally dehisce, so white tulle was placed around mother plants to prevent seed loss from controlled pollinations. Final pollinations at the apex of the plant were conducted without removing tulle from the lower inflorescences. Starting 4 February 2013 seed was collected using tweezers from tagged inflorescences only. Seed was placed into 15 ml conical vials and held at 4 °C until seed collection was finished on 28 February 2013.

On 16 March 2013, 144 seeds from each cross were sown in plug trays [288-cell size square (6 ml volume)] filled with 100% potting mix. Dark seeds, as opposed to light-colored seeds, were sown when available, as dark seeds are reported to have a much higher rate of germination (Goettemoeller and Ching, 1999). Seed harvested from crosses 2, 15 and 16 had no black seed. The seeded trays were placed into a chamber with a temperature of 23 °C, and day length of 16 h, supplied by fluorescent and incandescent lamps. Seeded trays were covered with clear plastic domes, and set on felt material dampened with reverse osmosis water.

Parent propagation and seedling culture. On 1 April 2013, at least 16 cuttings each of the eight genotypes selected as parents were taken and treated as described above, under the section *"Parent plant material and propagation".*

Cessation of use of plastic domes occurred on 8 April 2013, and hand-watering of seedlings began, supplying (mg L^{-1}): 62.5 N, 6 P, 50 K, 32.5 Ca, 6 Mg, 0.5 Fe and Cu, 0.25 Mn and Zn, 0.15 B, and 0.05 Mo (MSU RO Water Special, GreenCare Fertilizers, Inc., Kankakee, IL, USA). Seedlings were moved to a glass-covered greenhouse on 11 April 2013 under a 16 h photoperiod, controlled as described in Chapter 2, expt. 1, and an average temperature of 21.7 °C with standard deviation of 0.76 °C for the duration of plant growth. Seedlings were placed on capillary mats to prevent desiccation and continued to be hand-watered with the same fertilizer regime. Numbers of seedlings produced from each cross are reported in Table 2.

Progeny and rooted cuttings of parents ("rooted" indicates that roots reached into every corner of the plug cell) were transferred to 50-cell plug trays (76 mL cell volume) filled with 100% potting mix on 18 and 26 April 2013, respectively. Parent trays were randomized among progeny trays on the same bench. On 14 May 2013, both parents and progeny were repotted into

15-cm square plastic containers (2506 mL) filled with 100% potting mix. The individuals were randomized across greenhouse benches and spaced 30 cm mid-pot to mid-pot.

Although progeny plants remained consistently vegetative, some of the plants from all of the parent lines initiated flowering. When this occurred, plants were pinched back one node and treated with 200 mg L^{-1} ethephon. Ethephon was applied on three separate occasions to flowering individuals, 28 May and 17, 28 June 2013.

Tissue sampling and steviol glycoside analysis. On 3 July 2013, tissue samples were collected and prepared as described in Chapter 1, Experiment 1, with the exception that, after drying, samples were kept in collection envelopes and stored in silicone-sealed glass desiccators containing an absorbent consisting of Fuller's earth and dolomite (Dri-Rite Company, Chicago, IL USA). Samples selected for steviol glycoside analysis were prepared as described in Chapter 2, and stevioside and rebaudiosides A, C, D and X were quantified via ultrahigh performance liquid chromatography-tandem mass spectrometry. Six populations were selected for analysis based primarily on the diversity of the parents' glycoside profile, and secondarily on the size of the population as well as general health of the population (Table 3.3). Five of each of parental clones involved in the six populations selected for analysis were also analyzed.

Morphological data collection. On 8 July 2013, morphological data was collected for eight populations (Table 3.3), including height and number of nodes on the tallest branch in the same manner as described in Chapter 2, Expt. 1. Branch number (the number of branches greater than 15 cm in length emerging from the main stem), main stem caliper (the diameter in mm of the main stem directly above the soil line), leaf length (the length of the leaf in cm, from base to tip not including the petiole), and leaf width (the largest diameter of the same leaf) were also determined. For leaf dimensions, one leaf emerging from the sixth node down from the

apex of the main stem was measured. A disease index (scale of 1-10, where 1= 10% of plant affected, 2= 20% of plant affected, etc.), a vigor index (scale of 1-7, where 1 is a plant with small stature, small leaves and an unhealthy appearance not caused by disease, and 7 is very healthy, has great potential for biomass with abundant lateral branching supporting secondary branches) as well as whether or not the plant was flowering was also recorded. For the parents, only leaf length and width measurements were collected since they were vegetatively propagated.

Analysis of data. Frequency distributions from the six populations in which glycosides were quantified were generated for each of the concentrations of stevioside, and rebaudiosides A, C, D and X (Appendix). The concentrations were summed to calculate a total glycoside amount, from which percentage proportions were calculated for each glycoside, as done by Brandle (1999) with the exception that in this study rebaudiosides D and X were included in the total glycoside amount instead of dulcoside, which was not quantified in this study. Frequency distributions were also created for morphological characters from the same six populations (Appendix). All frequency distributions were created with SPSS version 19 (SPSS Inc., An IBM Company, Armonk, NY). Chi square analysis was performed for populations 13-01 and 13-14 which displayed two distinct classes of individuals in regards to glycoside profile. The classes for the chi square analysis differed for each glycoside, but were standardized in that each "low" class was four-fold smaller than the "high" class. Correlations were calculated using the Pearson's *r* statistic with SPSS. Independent t-tests were used to compare total glycoside content and morphological traits between plants in populations 13-01 and 13-14 that synthesized only stevioside with individuals in those populations that synthesized stevioside as well as downstream rebaudiosides.

Results

Trait distributions and segregation patterns. Two populations, 13-01 and 13-14 (which are reciprocal crosses), possessed some individuals with stevioside as the lone steviol glycoside produced (Figure 3.1). Other individuals in these populations produced multiple steviol glycosides, and the proportion of stevioside exhibited a bimodal distribution. All populations except for 13-16 exhibited transgressive segregation for stevioside percentage (Figure 3.1.)

Populations 13-01 and 13-14 were also unique rfor the percentage of rebaudioside A and rebaudioside C, as 25% of the individuals contained little to no rebaudioside A or rebaudioside C (Figures 3.2 and 3.3, respectively). These were the same individuals that possessed ca. 100% stevioside. For both populations, two groups could be distinguished, the "high" and "low" rebaudioside A and C individuals, which possessed more than 20% rebaudioside A and more than 5% rebaudioside C or less than 5% rebaudioside A and less than 1.25% rebaudioside C, respectively. Both populations segregated 3:1 for the high to low rebaudioside A ratio (Table 3.4), as well as 3:1 for the proportion of "high" to "low" rebaudioside C (Table 3.5). The other four populations displayed broad distributions of rebaudioside A, with transgressive segregation around both parent values, with the exception of population 13-16 (Figure 3.2). In the case of rebaudioside C, transgressive segregation occurred to the greatest extent in populations 13-01 and 13-14, and to a lesser extent in 13-13 and 13-08. Little to no transgressive segregation was observed in populations 13-10 and 13-16 (Figure 3.3.)

The percentage of rebaudiosides D and X was broadly distributed in populations 13-01, 13-13 and 13-14 (Figures 3.4 and 3.5). These three populations exhibited transgressive segregation for both glycosides, though the distributions were skewed towards the parent with the lowest proportion for both glycosides. Populations 13-08, 13-10 and 13-16 had no

transgressive segregation, and distributions for the percentage of rebaudiosides D and X were skewed towards the lower parent value. Populations 13-01 and 13-14 both segregated 3:1 for presence:absence of both rebaudiosides D and X, with numbers of individuals in agreement with the segregation of the percentage rebaudioside A. The criteria used for the "low" and "high" classes were less than 0.5% and greater than 2% rebaudioside D, and less than 0.1% and greater than 0.4% rebaudioside X, respectively.

Frequency distributions for total glycoside concentration were bimodal in three of the six populations, 13-01, 13-13, and 13-14. Transgressive segregation was present in every population, and always exceeded the highest parent value, especially those with distributions that were unimodal (Figure 3.6).

The parents for the six populations were selected based on rebaudioside D percentage when calculated from the rebaudioside A plus D total only. The frequency distributions for this trait were skewed towards the lowest parent value or even below the lowest parent value. Populations 13-01, 13-13 and 13-14 showed transgressive segregation beyond the highest parent value (Figure 3.7).

Correlation of traits: steviol glycosides.

Among the 301 individuals sampled for glycoside analysis, 21 individuals had little to no rebaudiosides A, C, D or X. These individuals were omitted from the main data set so as not to skew the correlations. The criteria used for omission was less than 1 mg/g rebaudiosides A and C. The individuals omitted were not significantly different from the included individuals from the same populations in regards to total glycosides, height, stem caliper, number of branches, leaf length and leaf width, vigor or disease incidence. The omitted individuals averaged fewer nodes, with a mean value of 15 nodes versus 19 nodes per plant (data not shown).

In many cases, trait correlations within a particular population differed from the response when all 280 individuals from the populations were pooled. For example, between stevioside and rebaudioside A, many populations had significant correlations but those correlations were opposite in direction. This also occurred between stevioside and rebaudioside D, rebaudiosides A and D, rebaudiosides A and X as well as rebaudioside D and total glycosides (Table 3.6).

The main data set containing 280 individuals had many significant correlations among the five glycosides quantified in this study. Overall, stevioside concentration was positively correlated with rebaudiosides A and C concentrations, as well as total glycoside concentration. Significant negative correlations were found between stevioside and rebaudiosides D and X concentrations (Table 3.6). The concentration of rebaudioside A was positively correlated with rebaudioside C and total glycoside concentration, yet negatively correlated with rebaudiosides D and X (Table 3.6). Rebaudioside C concentration was highly correlated with total glycoside concentration, however also negatively correlated with rebaudiosides D and X (Table 3.6). Rebaudioside D concentration was positively related to the concentration of rebaudioside X, yet both rebaudiosides D and X were negatively correlated to total glycoside concentration (Table 3.6). The percentage of rebaudioside D, when calculated from the total of rebaudiosides A and D, was negatively correlated with stevioside, rebaudiosides A, C and total glycoside concentration. Positive correlations were found between rebaudiosides D and X with the percentage of rebaudioside D calculated from the total of rebaudiosides A and D (Table 3.6.)

Correlation of traits: morphological and steviol glycosides.

For each plant which had both steviol glycoside quantification as well as various morphological traits measured (301 total), correlations using the Pearson's *r* statistic were performed. Stevioside concentration was positively correlated with stem caliper, number of

branches, leaf length and vigor. Node number was negatively correlated with stevioside concentration (Table 3.7). Rebaudiosides A and C had positive correlations with stem caliper, number of branches, vigor and leaf length (Table 3.7). Rebaudioside D was negatively correlated with leaf length, yet positively correlated with node number and height. Rebaudioside X was negatively correlated with stem caliper, number of branches, vigor, and leaf length. Total glycoside content was positively correlated with stem caliper, number of branches, vigor and leaf length (Table 3.7). The percentage of rebaudioside D, when calculated from the total of rebaudiosides A and D only, was negatively correlated with stem caliper, number of branches, vigor, and leaf length (Table 3.7).

Individual populations were examined to see if any were in disagreement with overall significant correlations. This was the case only with population 13-16, which had a significant negative correlation between rebaudioside D and node number, contrasting with the overall positive relationship (data not shown).

There were also significant correlations among morphological traits. Stem caliper was positively correlated with branch number, vigor and height. Branch number and vigor were highly correlated, and branch number was also correlated positively with number of nodes and height. Node number and height were also positively correlated with vigor, respectively. Leaf length and leaf width shared a positive correlation of (Table 3.7). Node number and height were slightly positively correlated. The incidence of disease was not significantly correlated with any of the glycoside or morphological traits measured (data not shown).

Discussion

Several stevia populations were developed among individuals varying in the proportion of rebaudioside D from the total amount of rebaudioside A and rebaudioside D (i.e. the biosynthetic efficiency of rebaudioside D from rebaudioside A, its immediate precursor) to examine the genetic control of rebaudioside D synthesis and the associations of rebaudioside D with other glycosides and with morphological traits. The proportions of other glycosides were maintained as similar as possible across genotypes in order to focus on the genetic control of rebaudioside D.

Correlations between different steviol glycosides exhibited a high degree of variability across populations. For example, although concentration of rebaudioside D was negatively correlated with the concentration of rebaudioside A when individuals in all populations were pooled and in populations 13-01 and 13-13, population 13-10 showed a positive correlation between the two compounds. For populations 13-08 and 13-16, rebaudioside D and total steviol glycosides were positively correlated, whereas a negative correlation was found overall and in population 13-13. Rebaudioside D was also positively correlated with stevioside concentration in population 13-16, while all other significant correlations between the two traits were negative. These contradictions demonstrate the genetic variability present in stevia, and specifically the variability present for traits relating to concentration and proportion of rebaudioside D. This variability could be utilized to better understand the genetic relationships between these traits, and as a resource for improving rebaudioside D-related traits in cultivated stevia.

The concentrations of stevioside and rebaudioside A were almost always positively correlated with total steviol glycosides (Table 3.6), while rebaudioside D was positively correlated with total steviol glycosides in only two populations. Negative correlations with total

steviol glycosides were found in one and three populations regarding rebaudioside D and rebaudioside X concentrations, respectively (Table 3.6). Stevioside and rebaudioside A are generally the glycosides produced in the largest concentrations, and rebaudioside A is thought to be the major precursor to rebaudioside D via the addition of a glucose on the C-19 hydroxyl group of rebaudioside A (Lee et al., 2013). However, rebaudioside A concentration was only positively correlated with rebaudioside D concentration in one population, 13-10. Therefore, increasing the concentration of the precursor molecule, rebaudioside A, does not necessarily directly result in increased concentrations of the downstream rebaudiosides D and X, demonstrating that synthesis of these compounds is regulated independently from rebaudioside A synthesis.

Several vigor traits, including stem caliper, branch number and leaf length were positively correlated with total steviol glycosides, stevioside and rebaudioside A (Table 3.7). However, the concentration of rebaudioside X and the percentage of rebaudioside D from the total of rebaudiosides A and D were negatively correlated to those same vigor traits. This indicates potential difficulty in developing stevia lines with high proportions of rebaudioside D and X that are also vigorous and productive with regard to biomass. Previous studies have shown that stevioside concentration is independent of yield and leaf: stem ratio (Brandle et al., 1992). Although neither trait was examined in this study, it would be interesting to examine both correlations with downstream analytes such as rebaudioside D or X.

In this study, rebaudiosides D and X were positively correlated and generally exhibited similar correlations with other glycosides. Glycosylation of rebaudioside A at C-19 to form rebaudioside D has been demonstrated *in vitro* to be mediated by the enzyme UGT91D2, (Kishore et al., 2011), which likely also mediates this reaction *in planta.* The synthesis of

rebaudioside X is thought to occur in a similar fashion when a single glucose is attached to the C-19 position of rebaudioside D, and could be mediated by the same enzyme. This is similar to the case of rebaudiosides A and C, which are also strongly positively correlated with each other and also share similar correlations with the other glycosides. The enzyme UGT76G1 is believed to be involved in the glycosylation steps synthesizing rebaudioside A from stevioside (Richman et al., 2005) as well rebaudioside C from dulcoside A (Brandle, 1999).

Two of the populations, 13-01 and 13-14 in this study, included individuals that produced less than 1 $mg \cdot g^{-1}$ rebaudiosides A, C, D and X, a characteristic not displayed by any of the parents. Stevioside was present in every plant assayed, and occasionally was the only glycoside present in detectable levels (such as the case with populations 13-01 and 13-14). In this study, populations 13-01 and 13-14 segregated 3:1 for the presence of rebaudiosides A, C, D and X, indicating that the presence of these glycosides is controlled by a single dominant gene. The segregation pattern is consistent with previous results by Brandle (1999), who examined the genetic control of the proportions of rebaudiosides A and C in two F_2 stevia populations. In the same study, Brandle speculated that for those individuals containing rebaudioside A, the actual proportion may be controlled by multiple loci or alleles. In support of this, individuals producing rebaudioside A in this study exhibited a continuous distribution for rebaudioside A proportion and displayed transgressive segregation beyond the parental values (Figure 3.2).

Rebaudiosides D and X followed the same segregation ratio as rebaudiosides A and C, with no recombinants. Rebaudioside D is thought to be synthesized largely from rebaudioside A, and since rebaudioside A is the direct upstream component found in the largest quantities, any appreciable amount of rebaudioside D likely results from the glycosylation of rebaudioside A. It

appears that the reactions mediated by the enzyme UGT76G1 are the major pathway steps determining the presence or absence of individual rebaudiosides.

Future stevia breeding efforts would benefit from studying the upstream activity in the steviol glycoside pathway when targeting more downstream analytes. For example, in this experiment parents were selected based on the variability in rebaudioside D only, however a fraction of the progeny resulted in little to no rebaudiosides A, D or X, suggesting a percentage of the offspring would be of no economic value if rebaudiosides A, D or X were the target product. The occurrence of stevia not producing glycosides of economic value could be avoided if parents were crossed initially to a "tester" line containing little to no rebaudiosides A, C, D and X (line homozygous for non-functional UGT76G1). Assuming that the presence of these glycosides is controlled by a single dominant gene (i.e. encoding UGT76G1), only homozygous dominant parents would produce offspring that always contain glycosides rebaudioside A, C, D and X. Only 50% of progeny would contain those glycosides if a heterozygous parent was crossed to a homozygous recessive parent. Following evaluation of the progeny from those crosses, classification of the parents for this trait could be used to design crosses that would produce progeny that all contained targeted glycosides of economic value. Since the results of this study show that most morphological traits, as well as total glycosides, are comparable between individuals containing nearly all stevioside versus individuals containing stevioside and other downstream glycosides, it would not be possible to identify one or the other with visual analysis. Determining the genetic status of the parents could also help ensure that costly analysis procedures are only carried out on individuals that could be of economic importance.

APPENDIX

Table 3.1. Steviol glycoside profiles for the eight genotypes used to select parents in a series of bi-parental crosses. Percentages of individual glycosides including stevioside, rebaudiosides A-D are reported, as well as the percentage of rebaudioside D calculated from the total of rebaudioside A and D only.

Parent	Stevioside (% of Total)	Reb. A (% of Total)	Reb. B (% of Total)	Reb. C (% of Total)	Reb. D (% of Total)	% Reb. D (Rebs. A + D total only)	Classification
11-486	23.7	49.5	4.2	12.7	9.9	16.7	$High_1$
10-34	22.4	41.1	5.3	13.9	17.3	29.6	$High_2$
11-596	33.2	47.2	2.0	10.9	6.8	12.6	High-Medium
11-616	15.5	63.4	3.6	10.2	7.4	10.4	Medium
11-574	31.3	51.1	2.9	11.3	3.4	6.2	Medium-Low
10-30	29.0	39.3	15.3	15.3	1.0	2.4	Low_1
10-24	19.0	57.8	8.2	13.2	1.8	3.1	Low_2
11-547	15.9	67.0	4.4	10.1	2.7	3.9	Low_3

The classification indicates whether that percentage of rebaudioside D is High (over 15%) High-Medium (12-15%), Medium (9-11%), Medium-Low (5-8%) or Low (less than 4%).

Table 3.2. Sixteen bi-parental crosses of *S. rebaudiana* designed around the variation of rebaudioside D percentage calculated from the rebaudioside A and rebaudioside D total.

Cross Designation	Type of Cross	Female Parent	Male Parent	Progeny No.
13-01	$High_1 \times High_2$	11-486	10-34	73
13-02	$High/Med \times High_2$	11-596	10-34	1
13-03	$Med \times High_2$	11-616	10-34	116
13-04	Med × High/Med	11-616	11-596	110
13-05	$Med/Low \times High_2$	10-34	11-574	103
13-06	Med/Low × High/Med	11-574	11-596	50
13-07	Med/Low × Med	11-574	11-616	89
13-08	$Low_1 \times High_2$	10-30	10-34	63
13-09	$Low_1 \times High/Med$	10-30	11-596	30
13-10	$Low_1 \times Med$	10-30	11-616	58
13-11	$Low_1 \times Med/Low$	10-30	11-574	0
13-12	$Low_1 \times Low_3$	10-30	11-547	1
13-13	$High_2 \times Low_3$	10-34	11-547	47
13-14	$High_2 \times High_1$	10-34	11-486	37
13-15	$Low_2 \times High_1$	10-24	11-486	5
13-16	$Low_2 \times Low_1$	10-24	10-30	19

Progeny numbers (Progeny No.) refer to the number of viable seedlings out of 144 seeds sown per cross.

Table 3.3. Populations selected for morphological and/or glycoside analyses, their parent identities, cross classifications and number of progeny.

Population	Female Parent	Male Parent	Cross Classification	Number of Progeny	Type of Data Collected
13-01	11-486	10-34	$High_1 \times High_2$	73	Morphological Glycoside
13-03	11-616	10-34	$Med \times High_2$	118	Morphological
13-05	10-34	11-574	$Med/Low \times High_2$	106	Morphological
13-08	10-30	10-34	$Low_1 \times High_2$	66	Morphological Glycoside
13-10	10-30	11-616	$Low_1 \times Med$	59	Morphological Glycoside
13-13	10-34	11-547	$High_2 \times Low_3$	47	Morphological Glycoside
13-14	10-34	11-486	$High_2 \times High_1$	37	Morphological Glycoside
13-16	10-24	10-30	$Low_2 \times Low_1$	19	Morphological Glycoside

Table 3.4. Observed and expected number of individuals and chi square analysis of proportions of rebaudioside A in two populations for 3:1 segregation.

Cross, Classification	"High" Reb A (Reb A > 20%)	"Low" Reb A (Reb A <5%)	df	χ^2	p-value
13-01, Observed	57	16			
13-01, Expected	54.25	18.25	1	0.409	0.54
13-14, Observed	32	5			
13-14, Expected	27.75	9.25	1	2.604	.11

P-value of greater than 0.05 indicates failure to reject null hypothesis, meaning observed probabilities are not significantly different than expected ones.

Table 3.5. Observed and expected number of individuals and chi square analysis of proportions of rebaudioside C in two populations for 3:1 segregation.

Cross, Classification	"High" Reb C (Reb C > 5%)	"Low" Reb C (Reb C <1.25%)	df	χ^2	p-value
13-01, Observed	57	14			
13-01, Expected	53.25	17.75	1	1.056	0.304
13-14, Observed	31	4			
13-14, Expected	26.25	8.75	1	3.438	0.064

P-value of greater than 0.05 indicates failure to reject null hypothesis, meaning observed probabilities are not significantly different than expected ones.

Table 3.6. Pearson correlation coefficients for 280 stevia individuals containing more than 1 mg/g rebaudiosides A and C. The values following 01 (13-01), 08 (13-08), 10 (13-10), 13 (13-13), 14 (13-14), 16 (13-16) indicate correlation coefficients for the individual populations.

	Reb. A (mg/g)	Reb. C (mg/g)	Reb. D (mg/g)	Reb. X (mg/g)	Total (mg/g)	Reb. D (% of Rebs. A+D)
Stevioside (mg/g)	13-01) .456**	13-01) .834**	13-01) -.191	13-01) -.690**	13-01) .870**	13-01) -.528**
	13-08) -.608**	13-08) .195	13-08) .215	13-08) -.664**	13-08) .672**	13-08) .470**
	13-10) -.398**	13-10) .571**	13-10) -.660**	13-10) -.774**	13-10) .664**	13-10) -.638**
	13-13) .586**	13-13) .853**	13-13) -.448**	13-13) -.800**	13-13) .907**	13-13) -.600**
	13-14) .182	13-14) .521**	13-14) -.356*	13-14) -.736**	13-14) .811**	13-14) -.458**
	13-16) .515*	13-16) .756**	13-16) .510*	13-16) -.155	13-16) .838**	13-16) -.132
Reb. A (mg/g)		13-01) .739**	13-01) -.270*	13-01) -.184	13-01) .830**	13-01) -.700**
		13-08) .481**	13-08) -.122	13-08) .663**	13-08) .171	13-08) -.584**
		13-10) .367**	13-10) .628**	13-10) .614**	13-10) .416**	13-10) .296*
		13-13) .899**	13-13) -.548**	13-13) -.485**	13-13) .869**	13-13) -.803**
		13-14) .868**	13-14) -.109	13-14) -.059	13-14) .715**	13-14) -.618**
		13-16) .876**	13-16) .319	13-16) .487*	13-16) .897**	13-16) -.664**
Reb. C (mg/g)			13-01) -.233	13-01) -.461**	13-01) .939**	13-01) -.687**
			13-08) -.011	13-08) .157	13-08) .724**	13-08) -.238
			13-10) -.103	13-10) -.194	13-10) .890**	13-10) -.304*
			13-13) -.542**	13-13) -.697**	13-13) .983**	13-13) -.767**
			13-14) -.155	13-14) -.412*	13-14) .890**	13-14) -.616**
			13-16) .374	13-16) .262	13-16) .952**	13-16) -.531*

Table 3.6 (cont'd).

	Reb. A (mg/g)	Reb. C (mg/g)	Reb. D (mg/g)	Reb. X (mg/g)	Total (mg/g)	Reb. D (% of Rebs. A+D)
Reb. D (mg/g)				13-01) .343** 13-08) .274* 13-10) .868** 13-13) .565** 13-14) .554** 13-16) .362	13-01) -.205 13-08) .248* 13-10) -.099 13-13) -.510** 13-14) -.225 13-16) .490*	13-01) .769** 13-08) .865** 13-10) .918** 13-13) .882** 13-14) .815** 13-16) .448
Reb. X (mg/g)					13-01) -.497** 13-08) -.147 13-10) -.203 13-13) -.712** 13-14) -.522** 13-16) .252	13-01) .301* 13-08) -.113 13-10) .744** 13-13) .544** 13-14) .464** 13-16) -.109
Total Glycosides (mg/g)						13-01) -.681** 13-08) .118 13-10) -.338** 13-13) -.751** 13-14) -.621** 13-16) -.467**

** indicates significance at 0.01 level (2-tailed), * is significance at 0.05 level (2-tailed). Total glycosides (Total) include stevioside, and rebaudiosides A,C,D and X.

Table 3.7. Pearson correlation coefficients for stevia plants involving morphological characteristics. Morphological by glycoside correlations calculated from the 280 stevia individuals containing more than 1 mg/g rebaudiosides A and C. Morphological by morphological correlations calculated from all stevia individuals (301). Total glycosides include stevioside, and rebaudiosides A,C,D and X.

	Stem Caliper	Branch Num.	Vigor	Leaf Width	Leaf Length	Node Num.	Height
Stevioside (mg/g)	.396**	.254**	.241**	.029	.150*	-.133**	.069
Reb. A (mg/g)	.297**	.229**	.239**	.034	.286**	.-.002	-.021
Reb. C (mg/g)	.470**	.354**	.401**	-.004	.205**	-.057	.052
Reb. D (mg/g)	-.094	-.100	-.093	.060	-.313**	.138*	.160**
Reb. X (mg/g)	-.233**	-.168**	-.195**	-.077	-.195**	.111	.105
Total (mg/g)	.449**	.311**	.368**	.038	.243**	-.081	.053
Reb. D (% of Rebs. A+D)	-.261**	-.203**	-.214**	.039	-.342**	.100	.071
Stem Caliper		.541**	.622**	.066	.060	.091	.273**
Branch Num.			.808**	-.097	-.081	.131*	.279**
Vigor				-.081	-.003	.136*	.362**
Leaf Width					.512**	.077	.011
Leaf Length						.102	-.083
Node Num.							.189**

** indicates significance at 0.01 level (2-tailed), * is significance at 0.05 level (2-tailed).

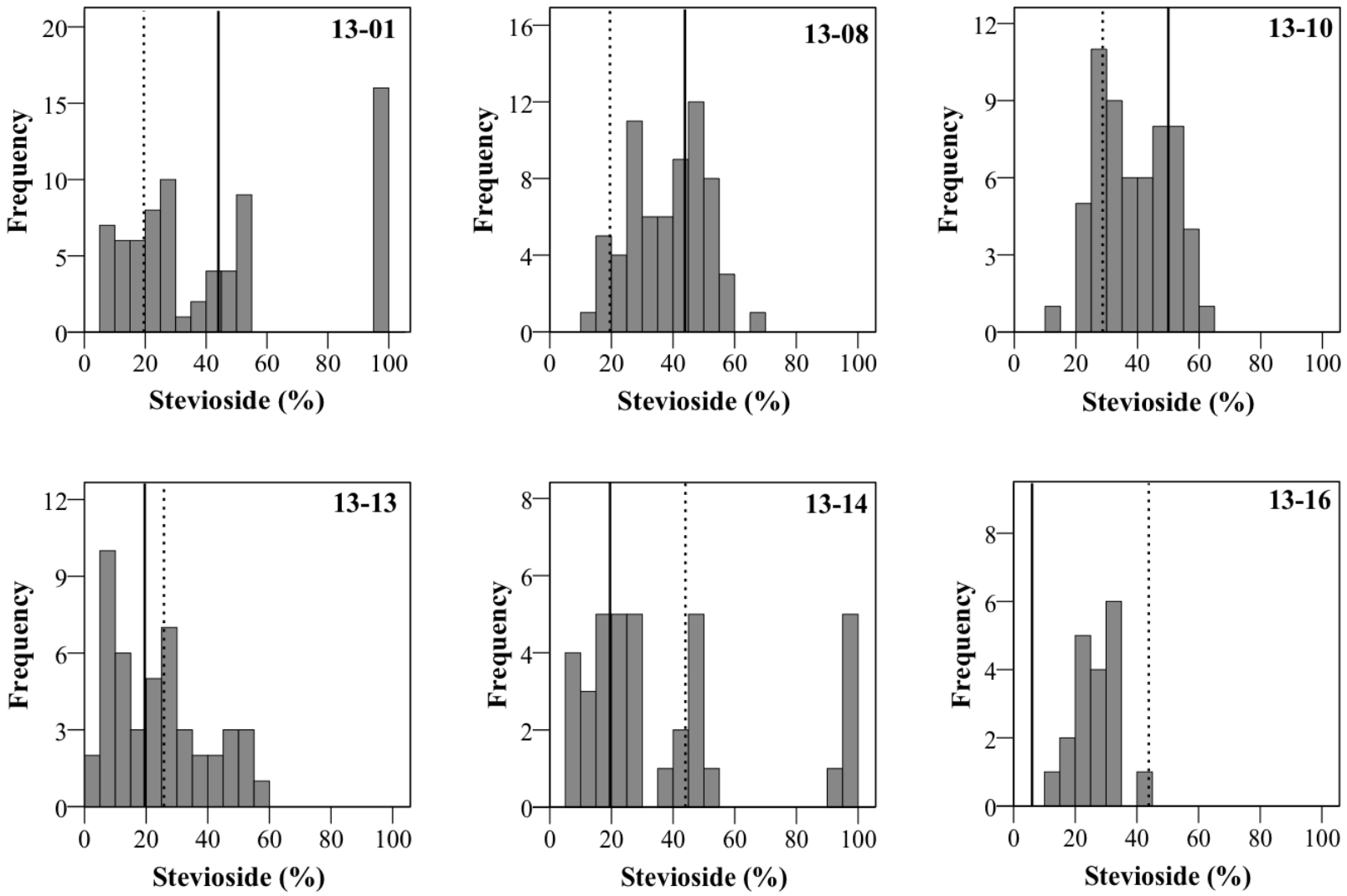

Figure 3.1. Frequency distributions for the proportion of stevioside for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

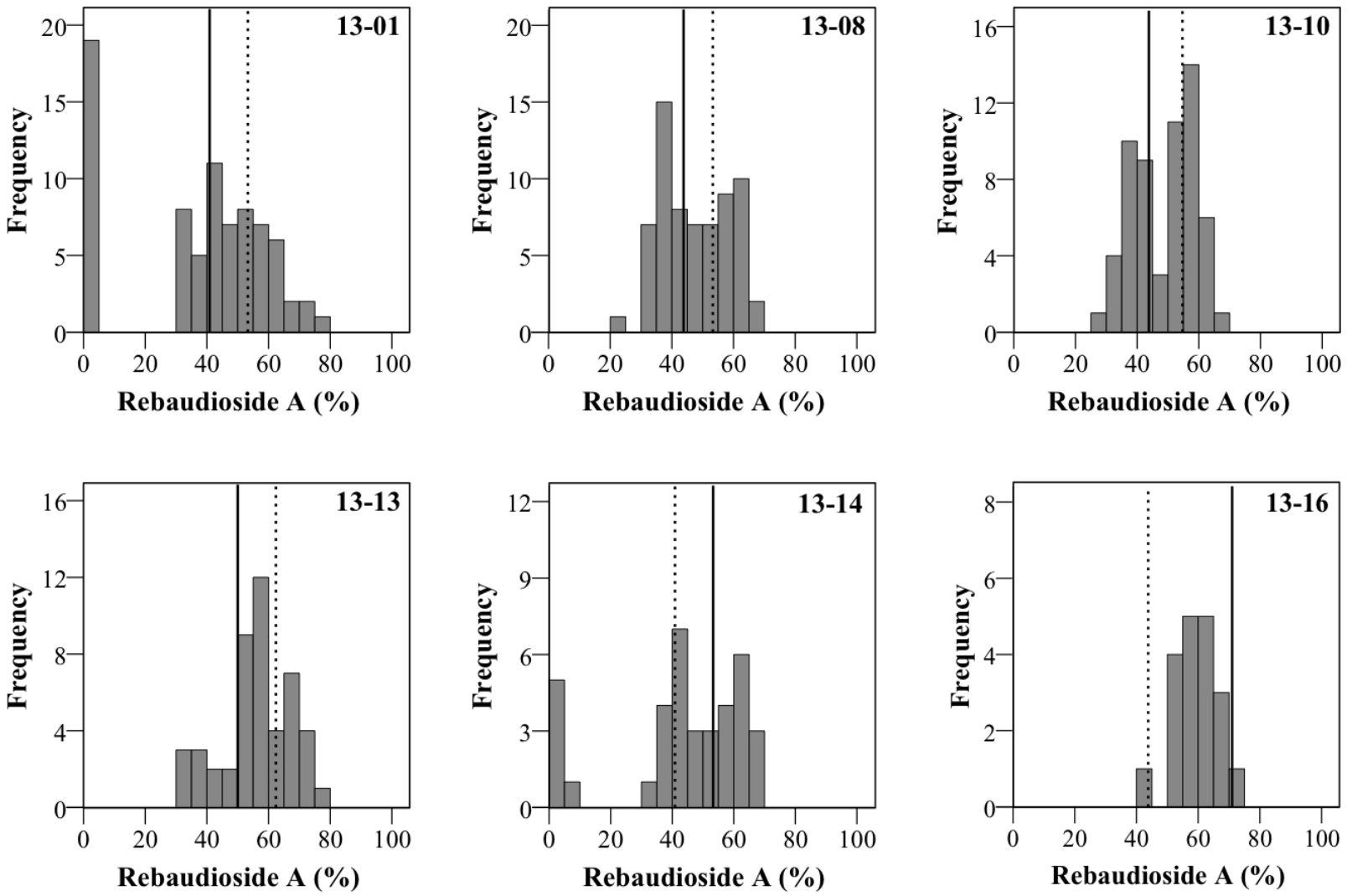

Figure 3.2. Frequency distributions for the proportion of rebaudioside A for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

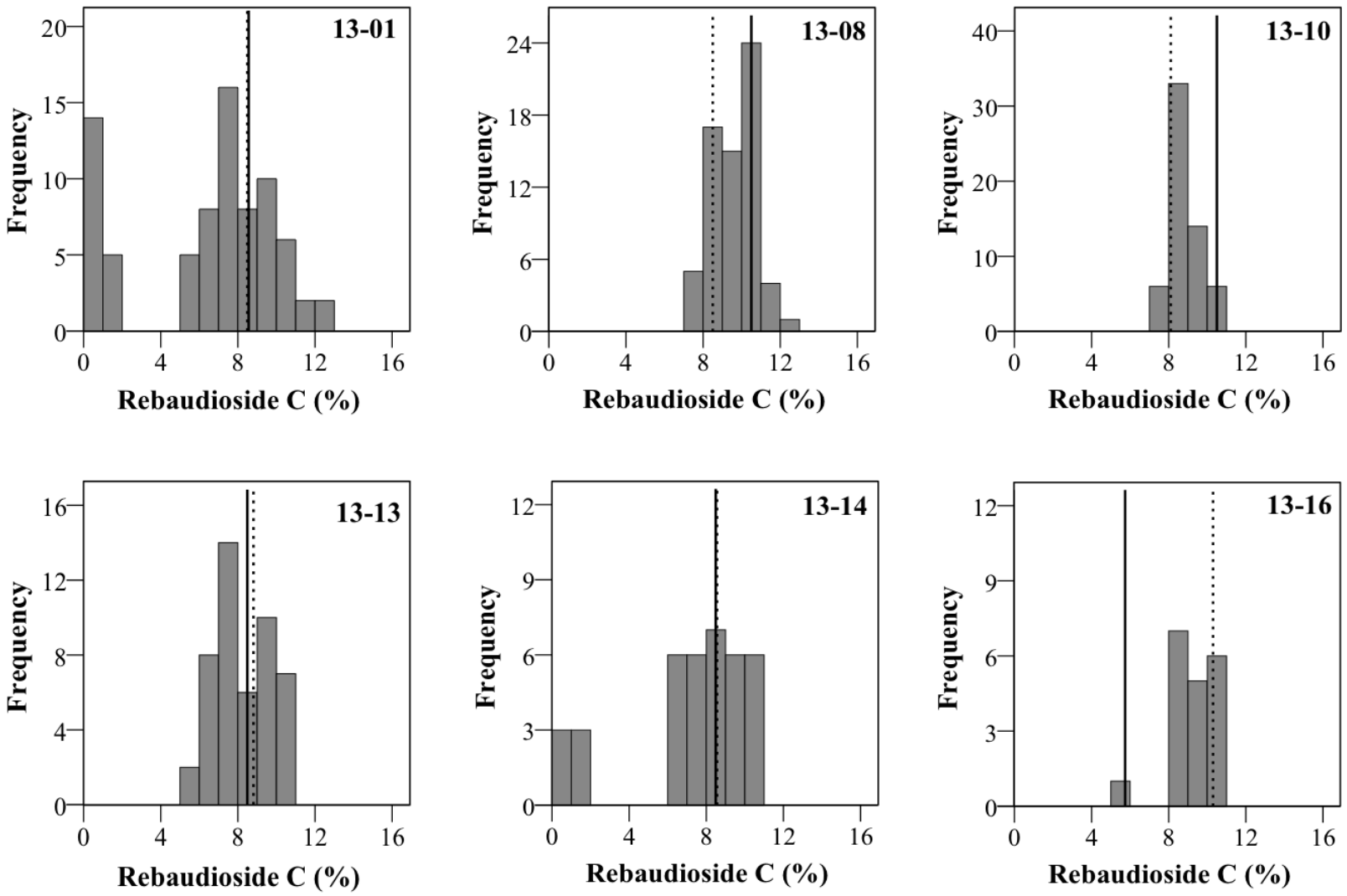

Figure 3.3. Frequency distributions for the proportion of rebaudioside C for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

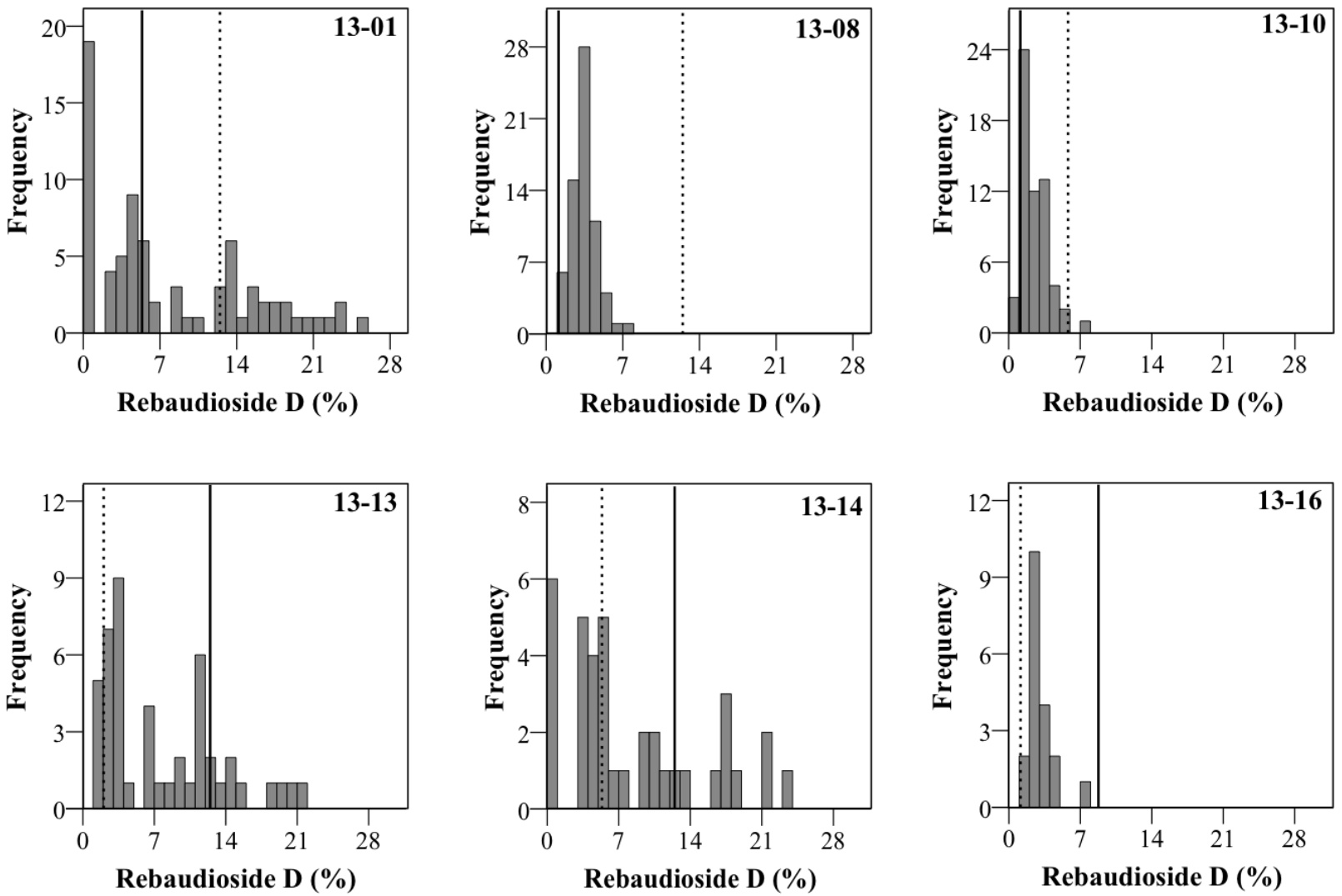

Figure 3.4. Frequency distributions for the proportion of rebaudioside D for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

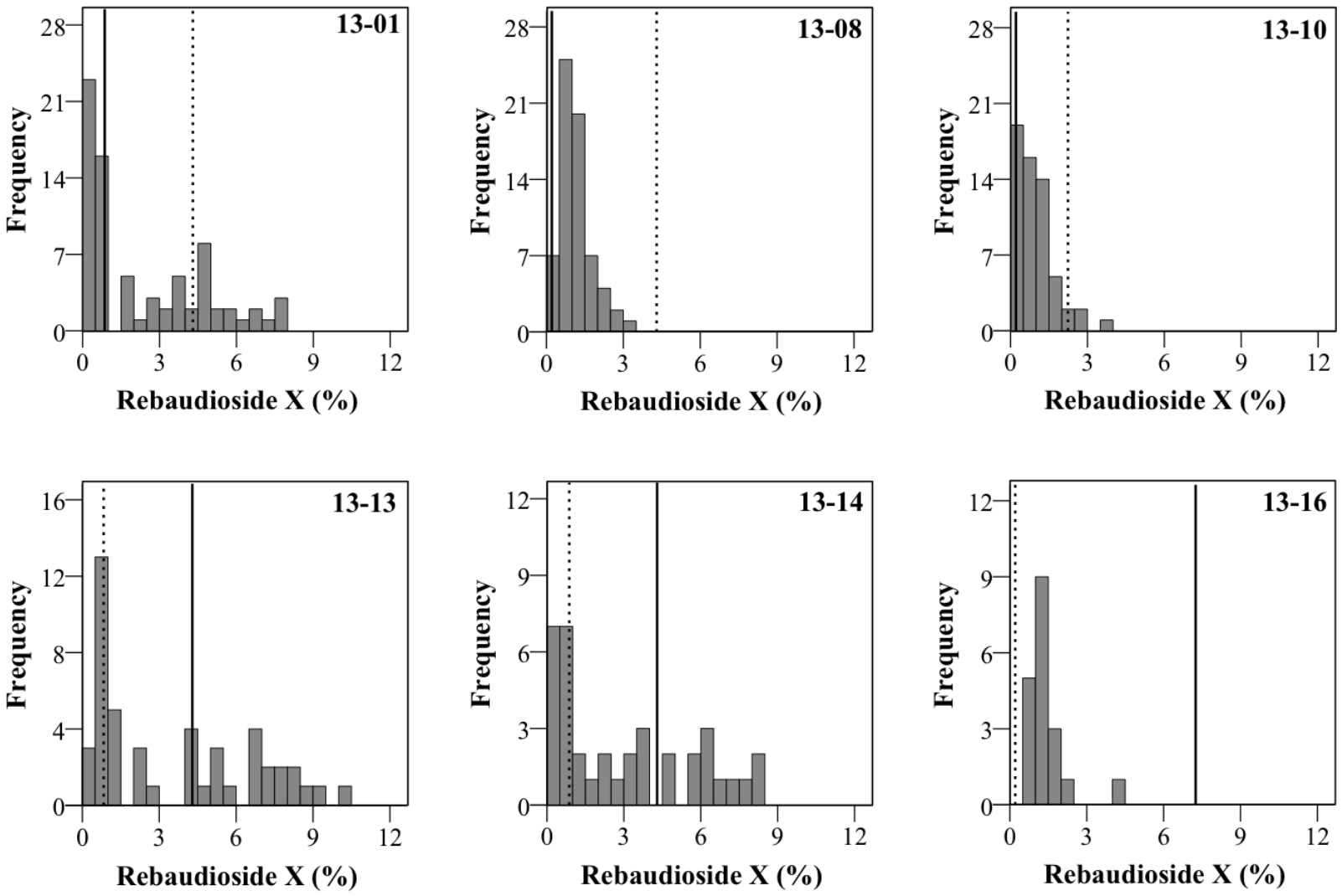

Figure 3.5. Frequency distributions for the proportion of rebaudioside X for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

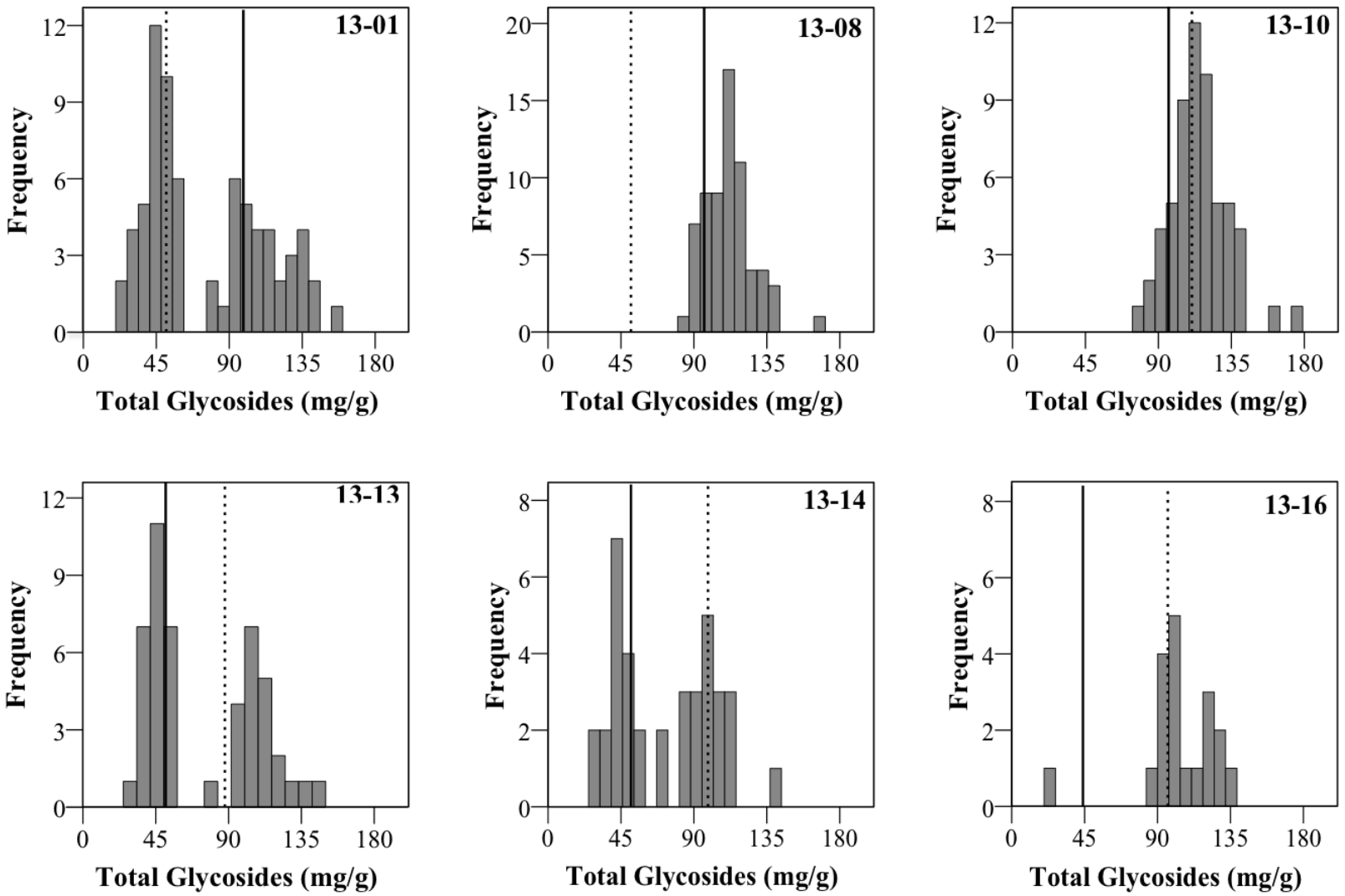

Figure 3.6. Frequency distributions for the total concentration of steviol glycosides including stevioside, and rebaudiosides A, C, D and X for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

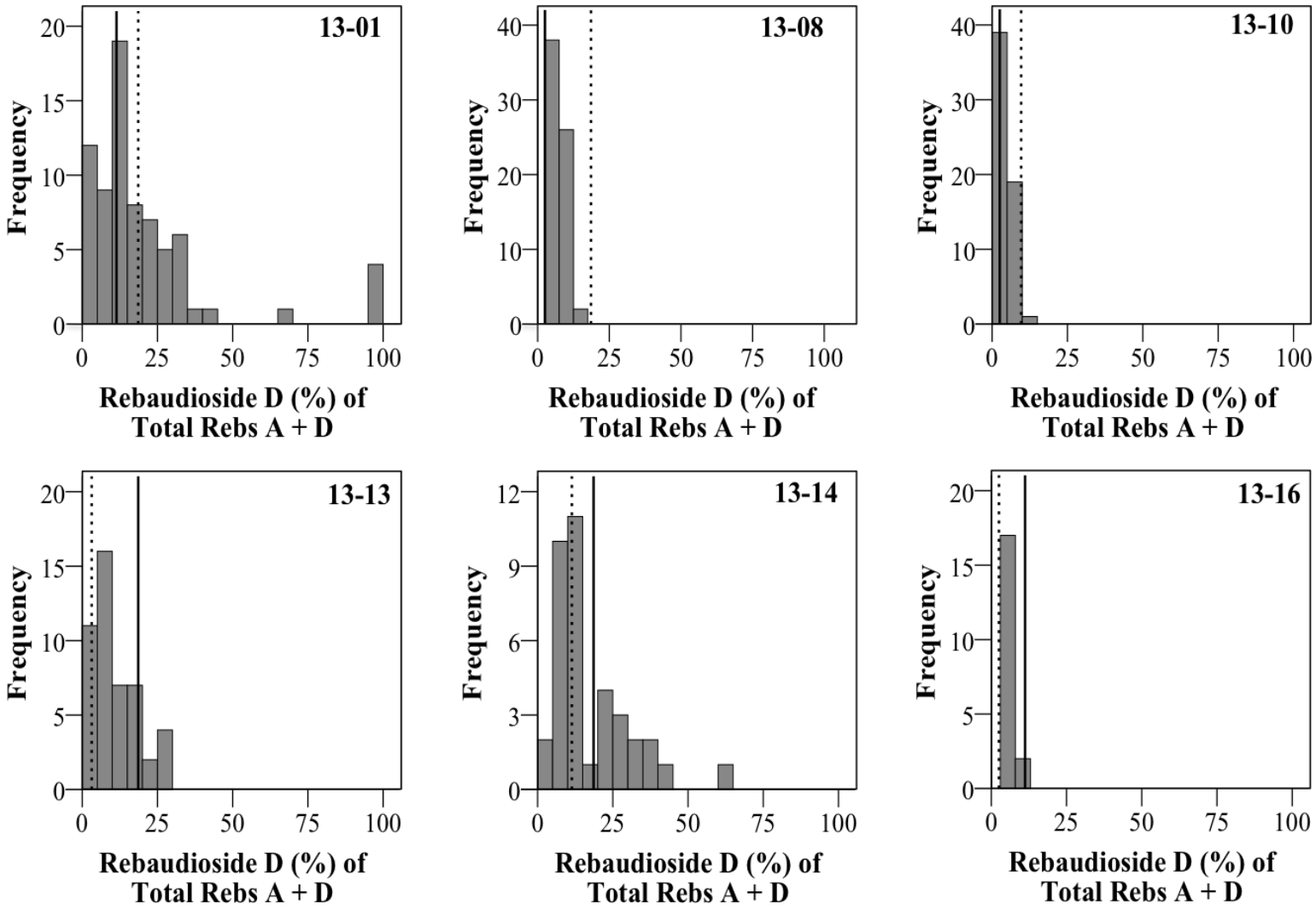

Figure 3.7. Frequency distributions for the percentage of rebaudioside D from the total composed of the two glycosides, rebaudiosides A & D for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

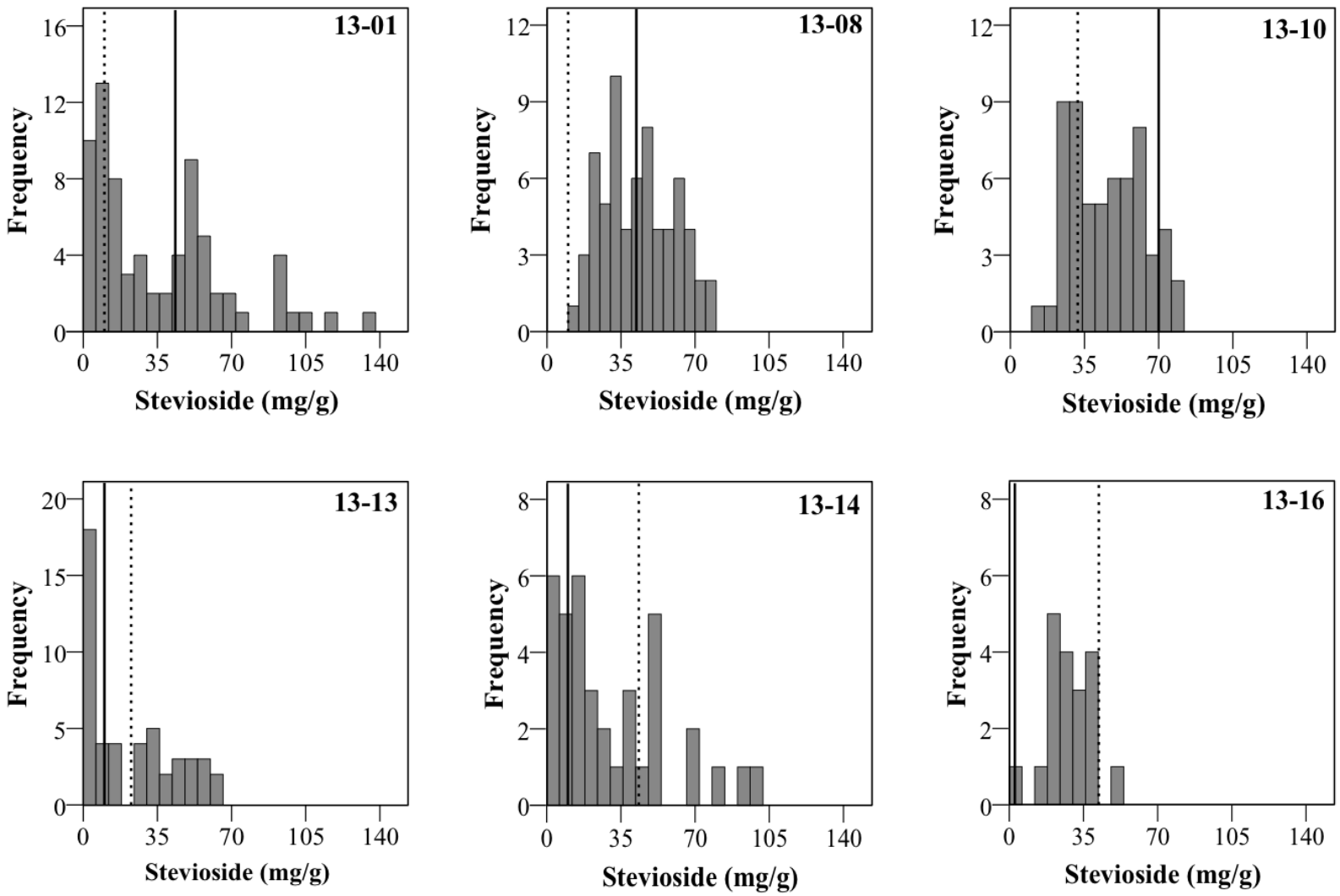

Figure 3.8. Frequency distributions for the concentration (mg/g) of stevioside for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

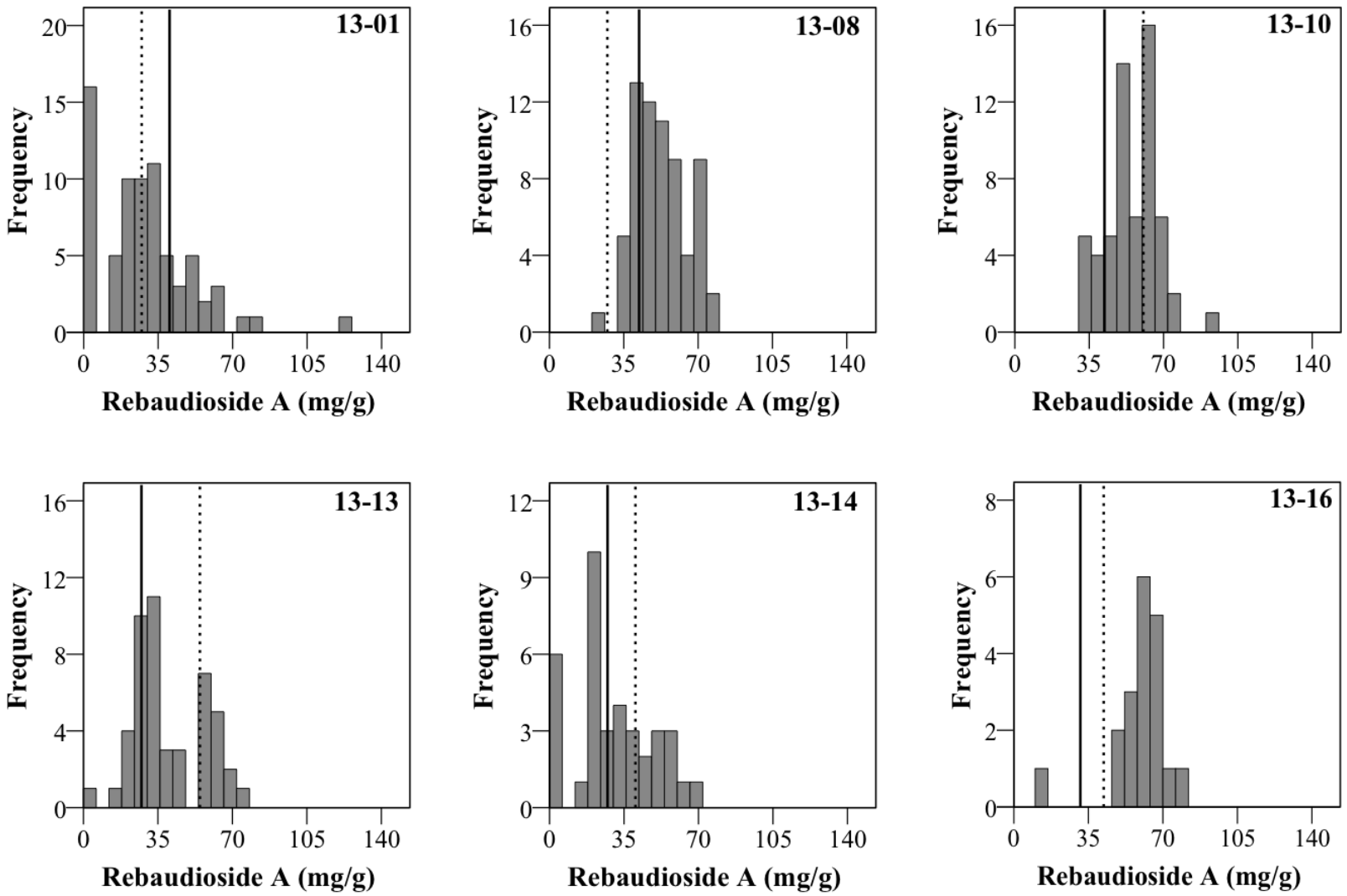

Figure 3.9. Frequency distributions for the concentration (mg/g) of rebaudioside A for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

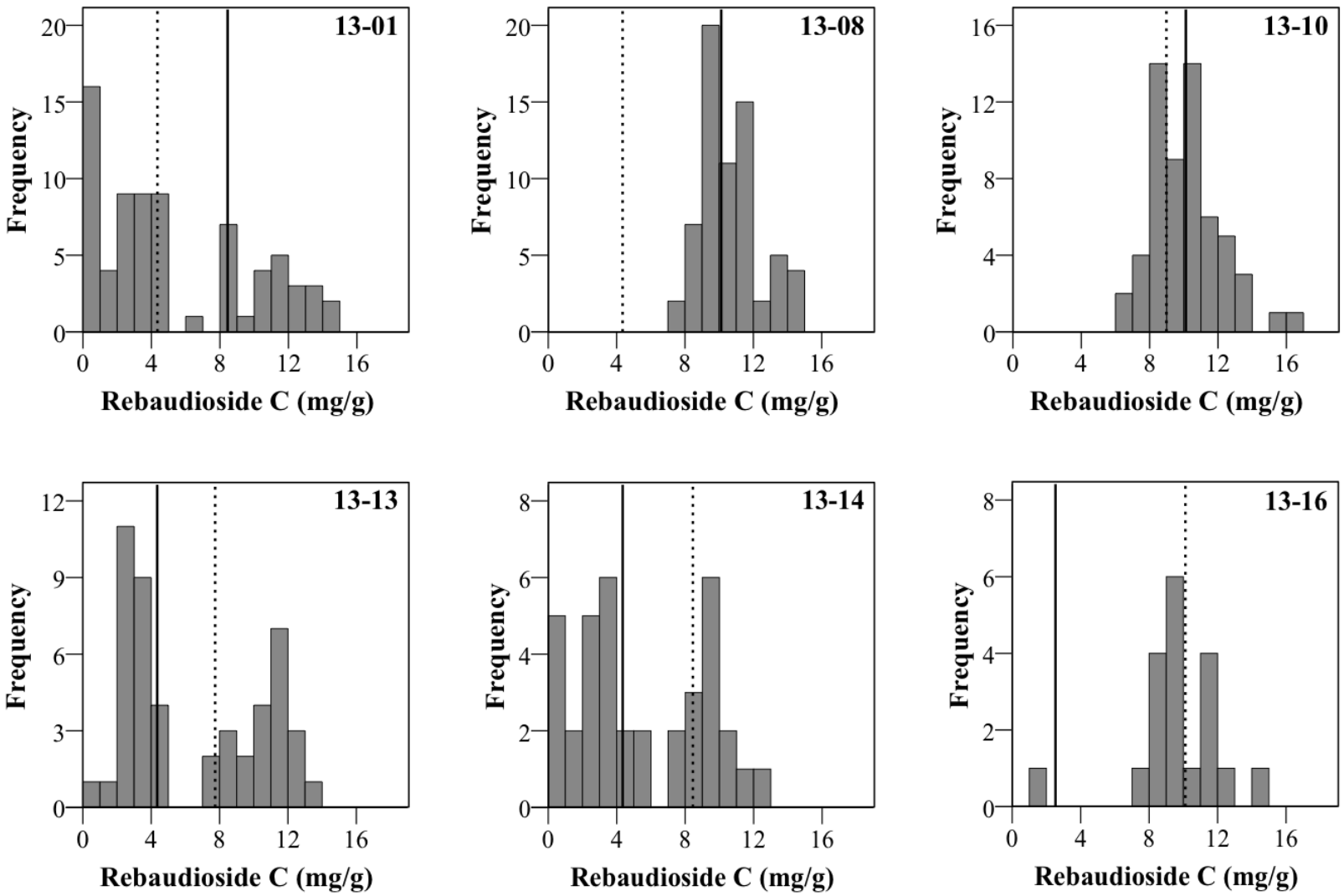

Figure 3.10. Frequency distributions for the concentration (mg/g) of rebaudioside C for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

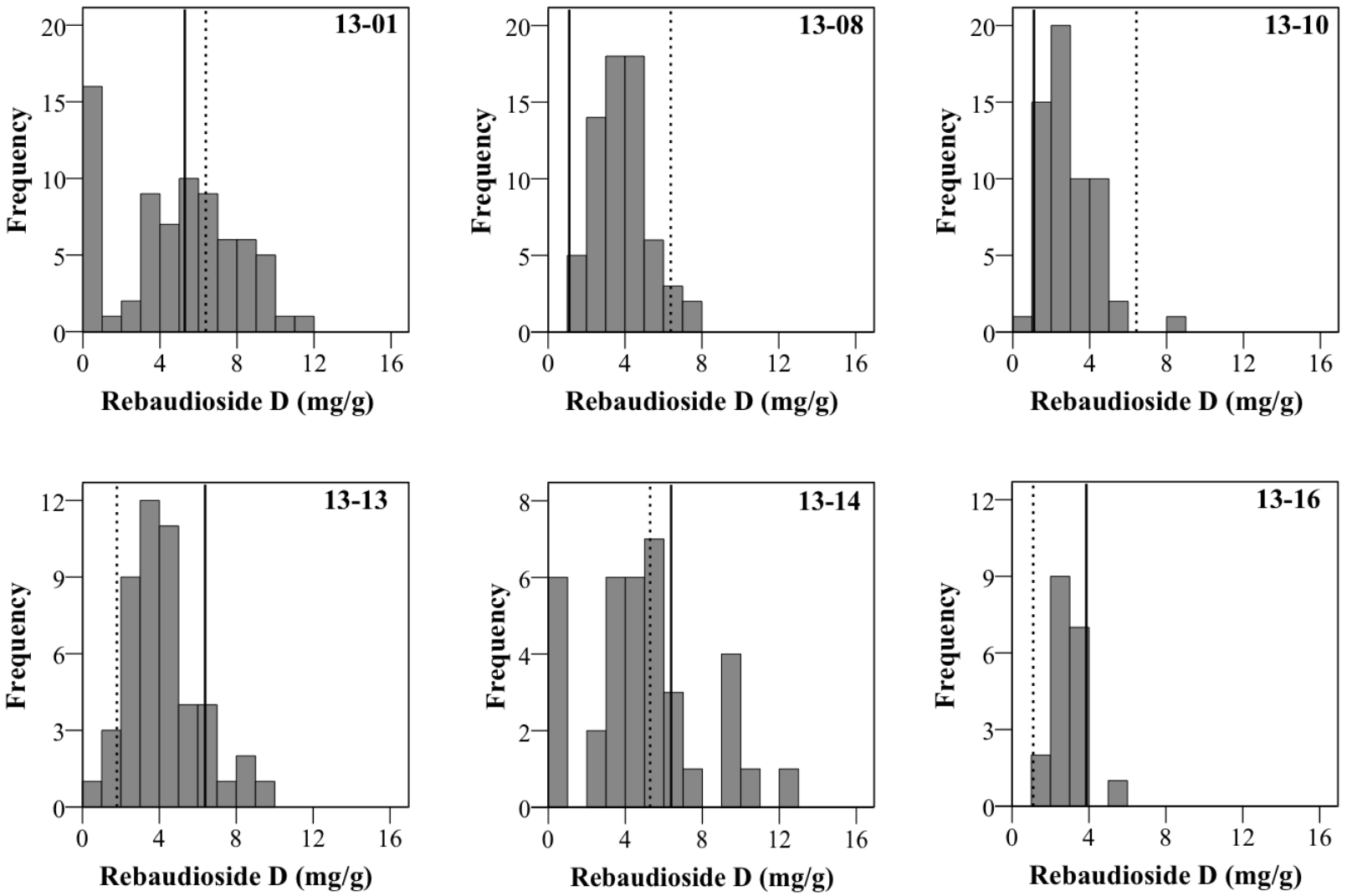

Figure 3.11. Frequency distributions for the concentration (mg/g) of rebaudioside D for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

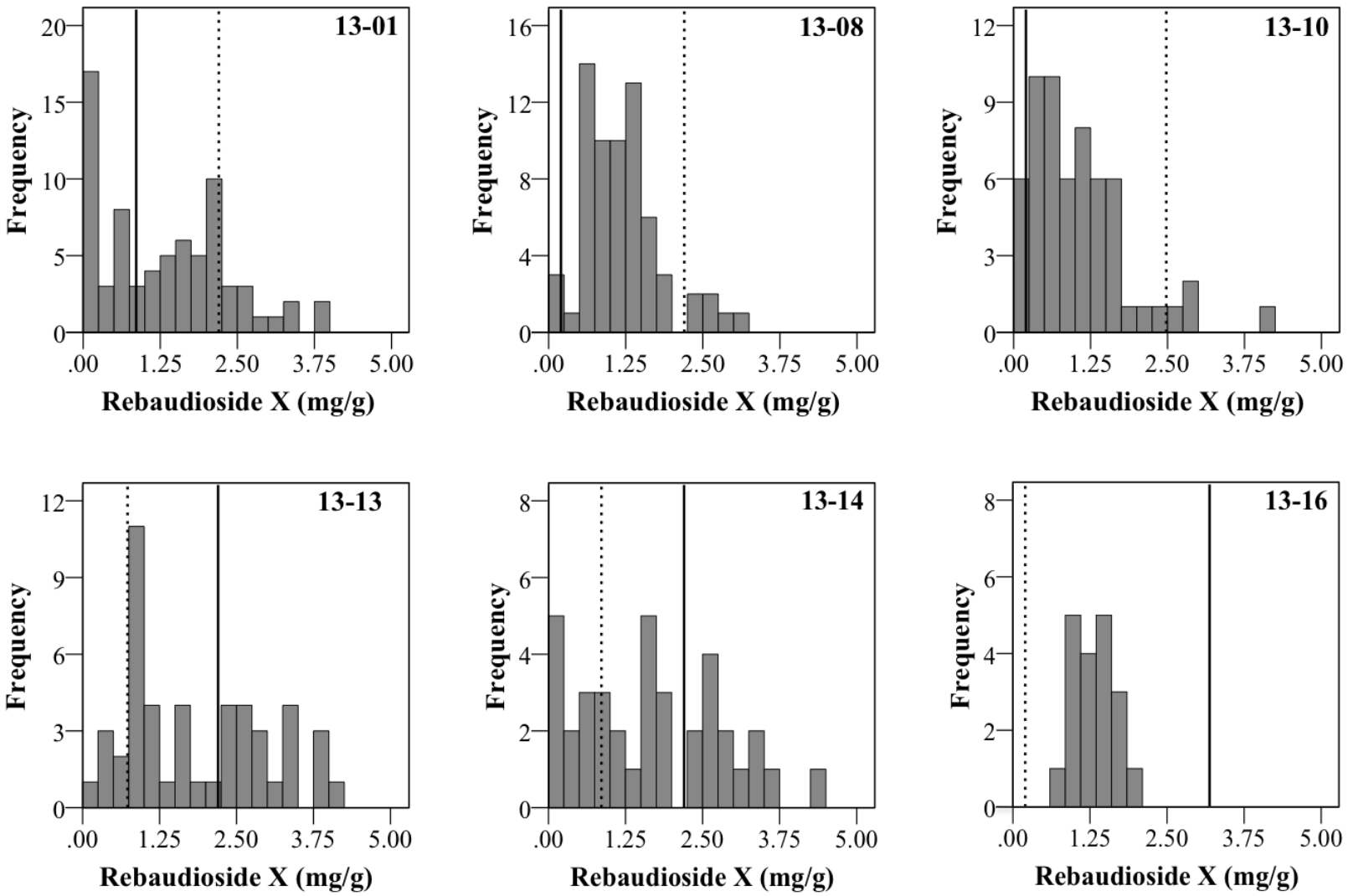

Figure 3.12. Frequency distributions for the concentration (mg/g) of rebaudioside X for six populations as described in Table 3.3. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

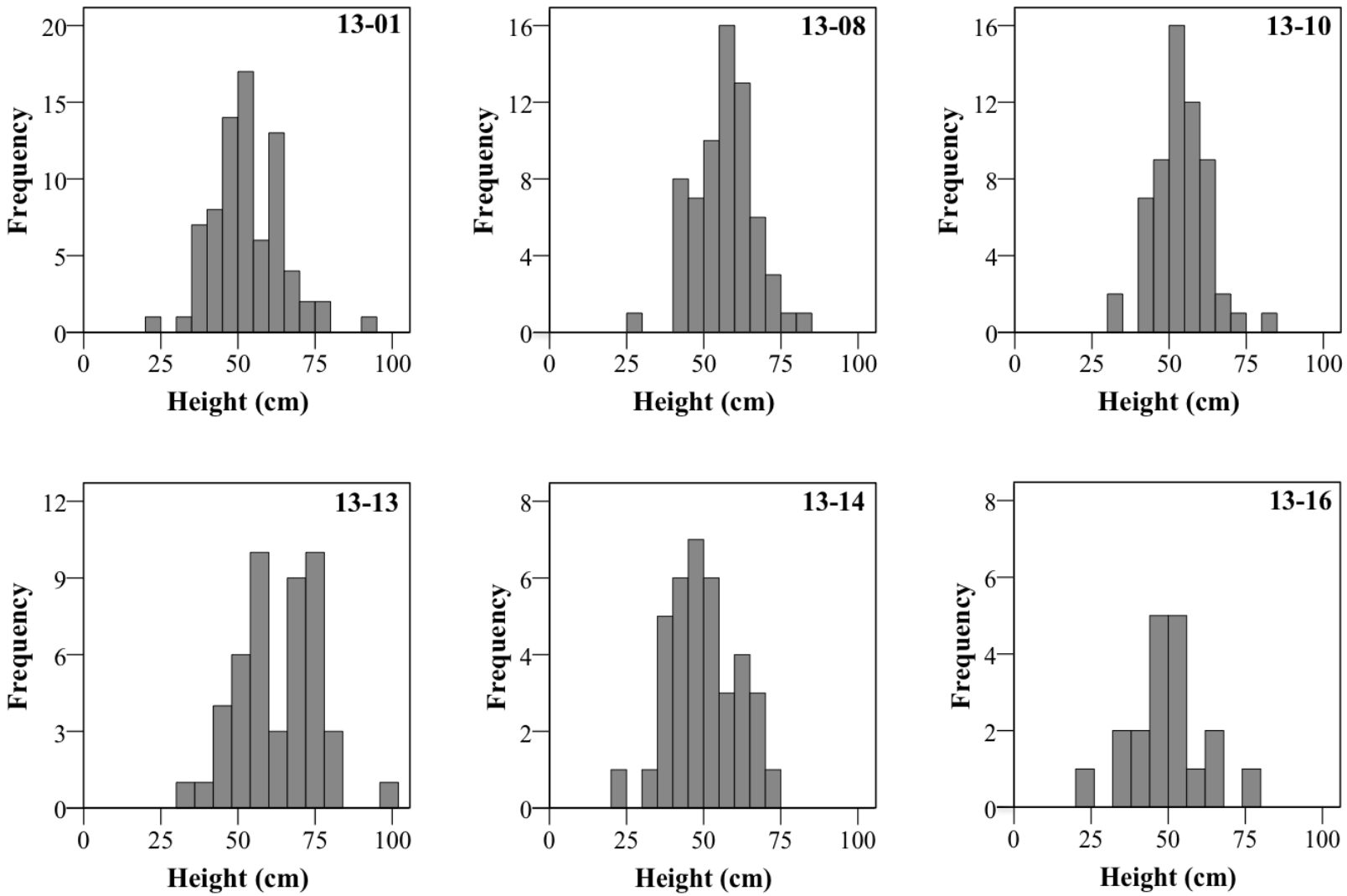

Figure 3.13. Frequency distributions of the height (cm) for individuals composing six populations as described in Table 3.3. Height was recorded as the distance (cm) of the furthest point of the canopy from the soil line.

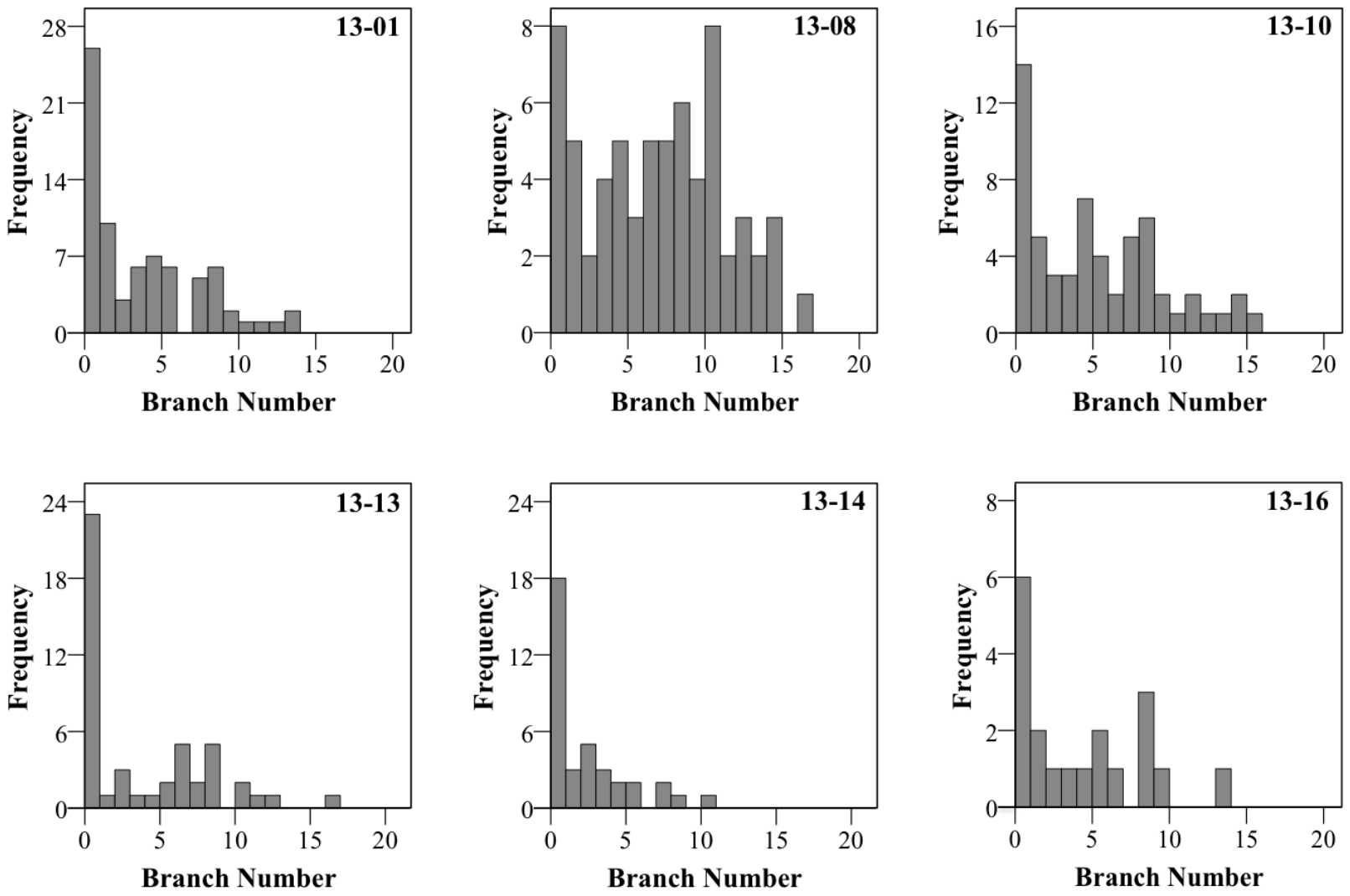

Figure 3.14. Frequency distributions of the number of branches for individuals composing six populations as described in Table 3.3. Branch number was a count of all branches that arise from the main stem that are longer than 15 cm in length. The main stem was not counted.

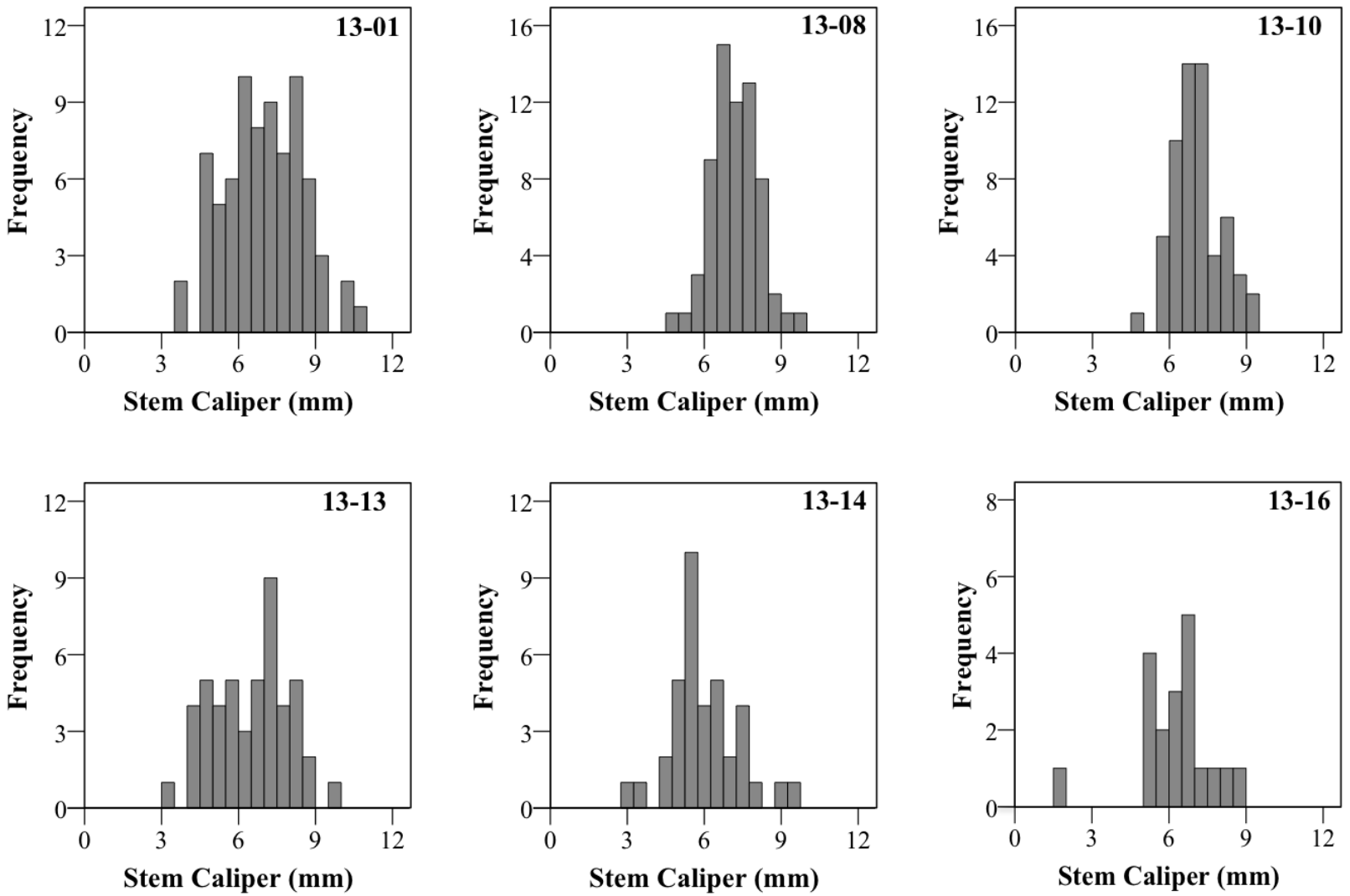

Figure 3.15. Frequency distributions for the diameter of the main stem caliper for individuals composing six populations as described in Table 3.3. Main stem caliper is the measurement (mm) of the diameter of the main stem, directly above the soil line.

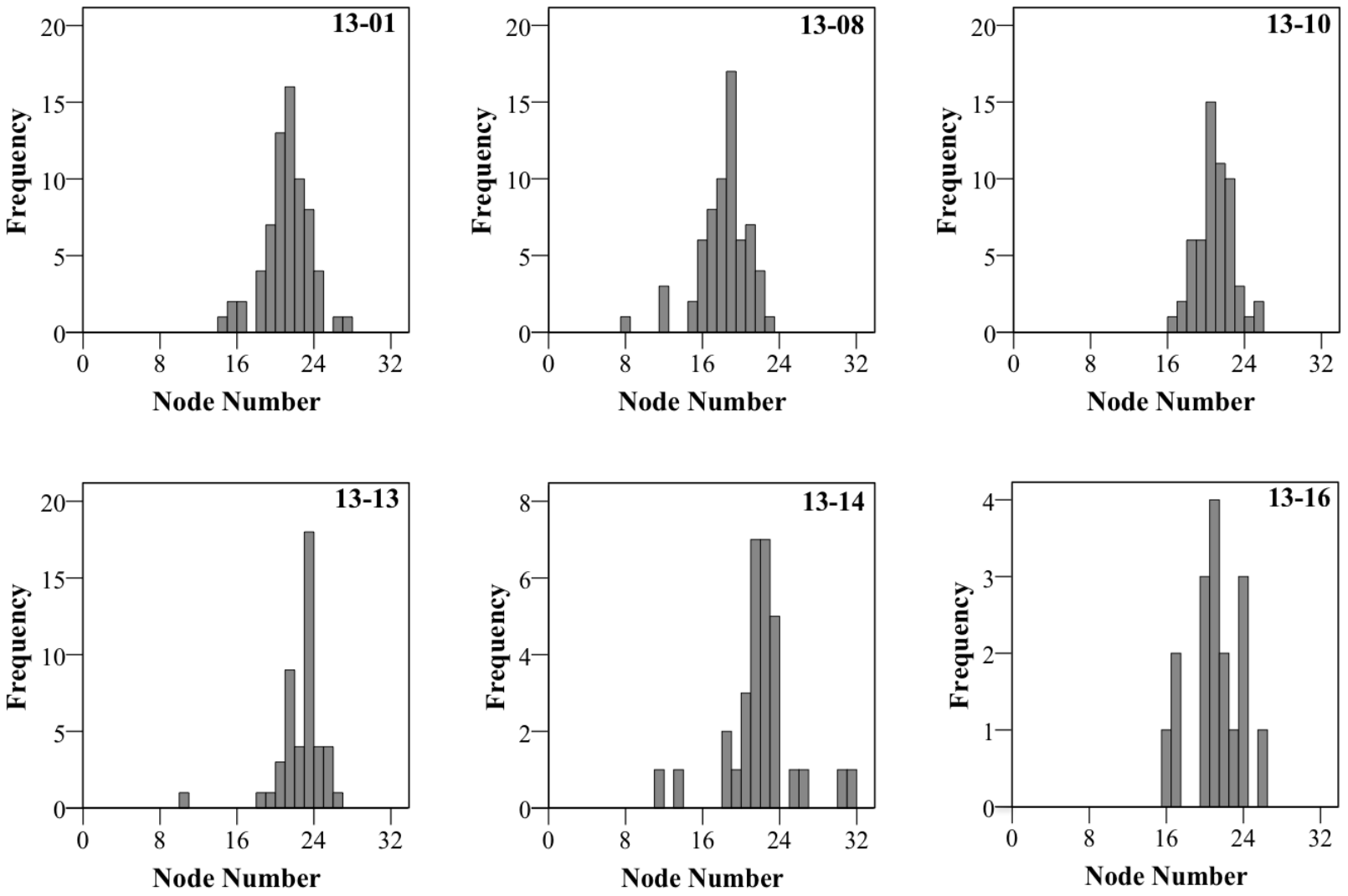

Figure 3.16. Frequency distributions for the number of nodes on the main stem for individuals composing six populations as described in Table 3.3. Node number was counted on the tallest branch, with opposite and alternate nodes each counted as one.

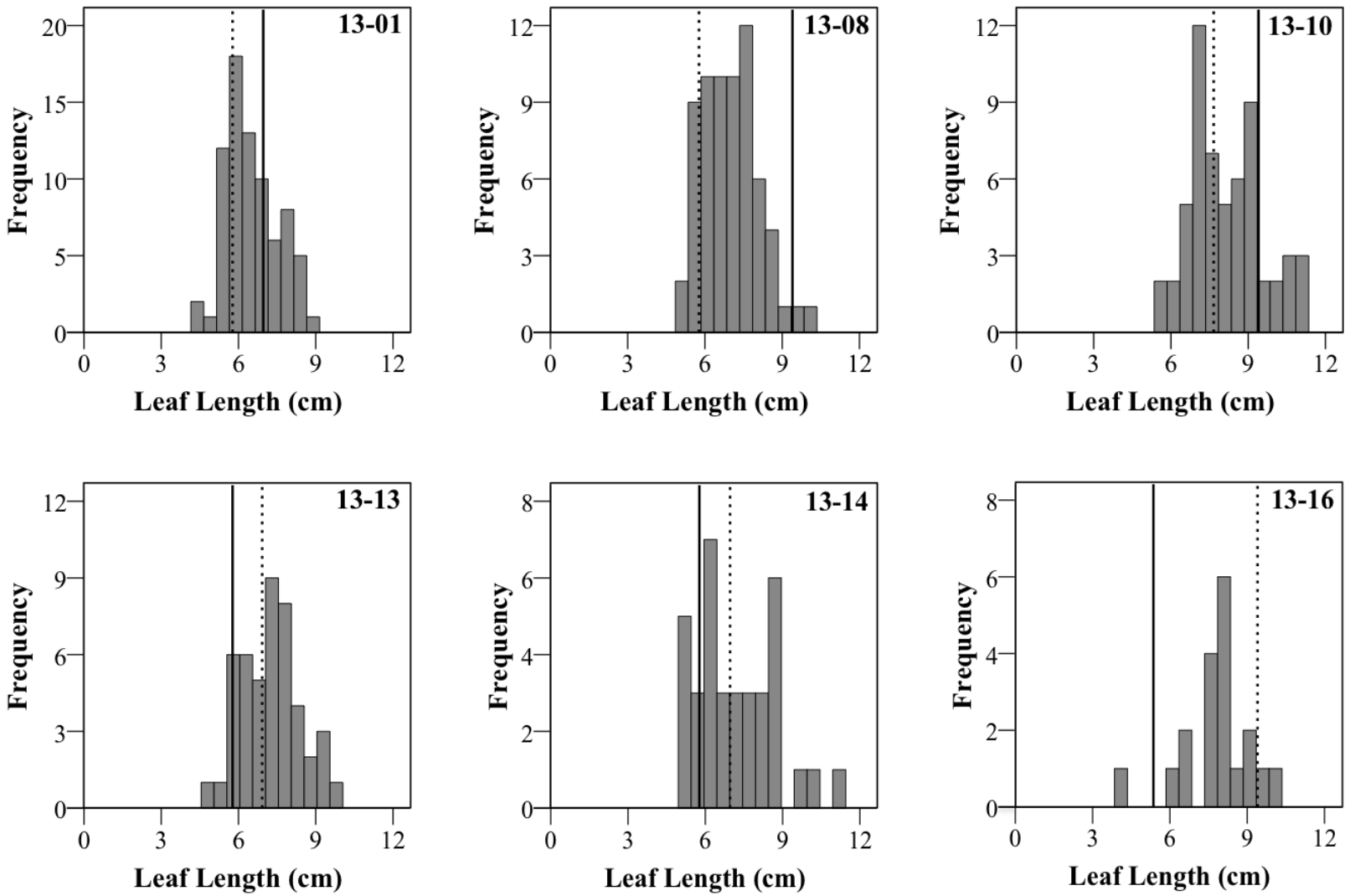

Figure 3.17. Frequency distributions of leaf length for individuals composing six populations as described in Table 3.3. Leaves measured were standardized as one leaf from the 6th node counting from top of main stem and measured (in cm) from base to tip not including the petiole. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

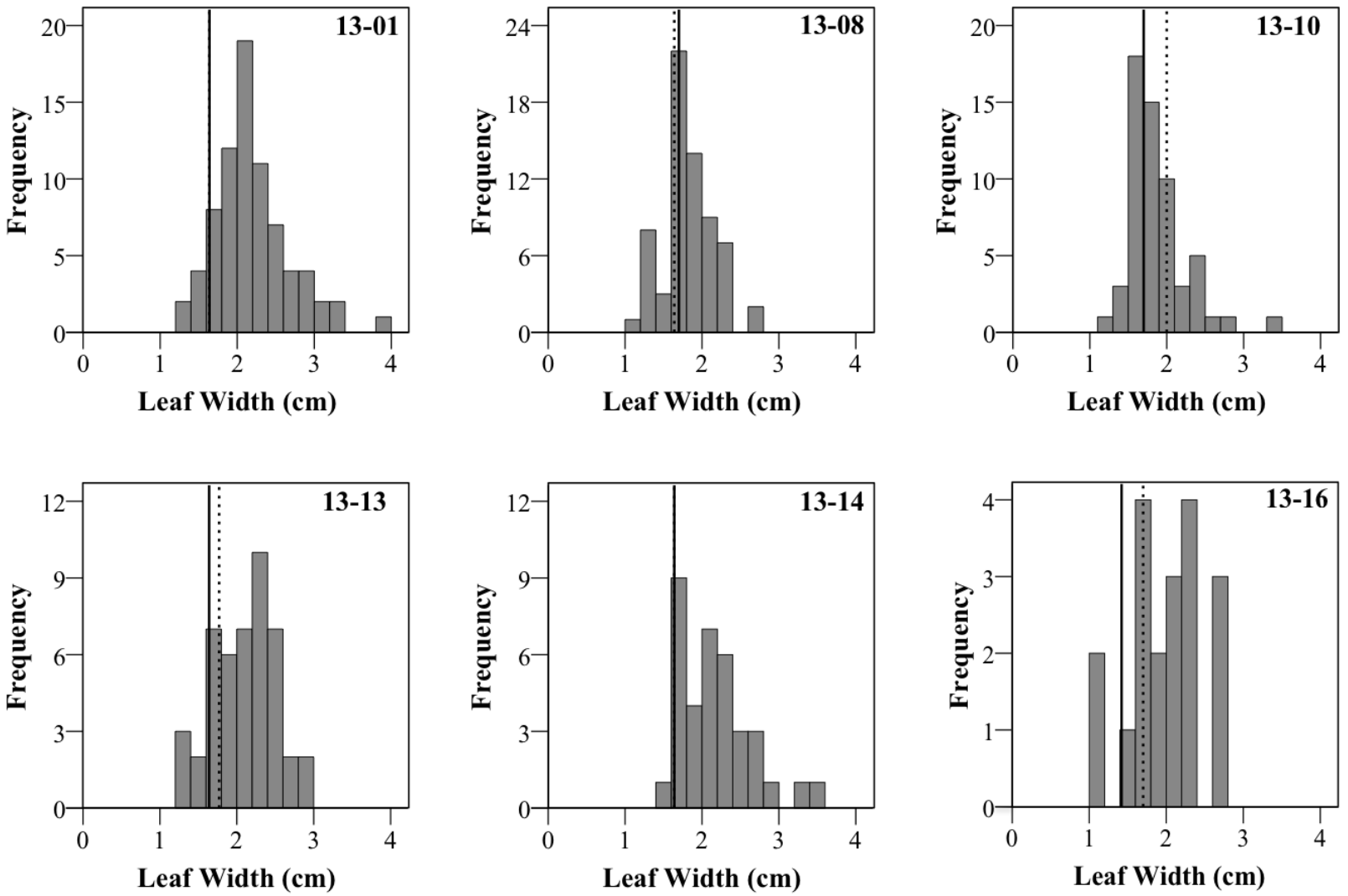

Figure 3.18. Frequency distributions of leaf width for individuals composing six populations as described in Table 3.3. The leaf measured was standardized as one of the leaves from the 6th node counting from top of main stem and measured (in cm) at the largest diameter. Reference lines in each distribution indicate the maternal (solid line) and paternal (dashed line) values.

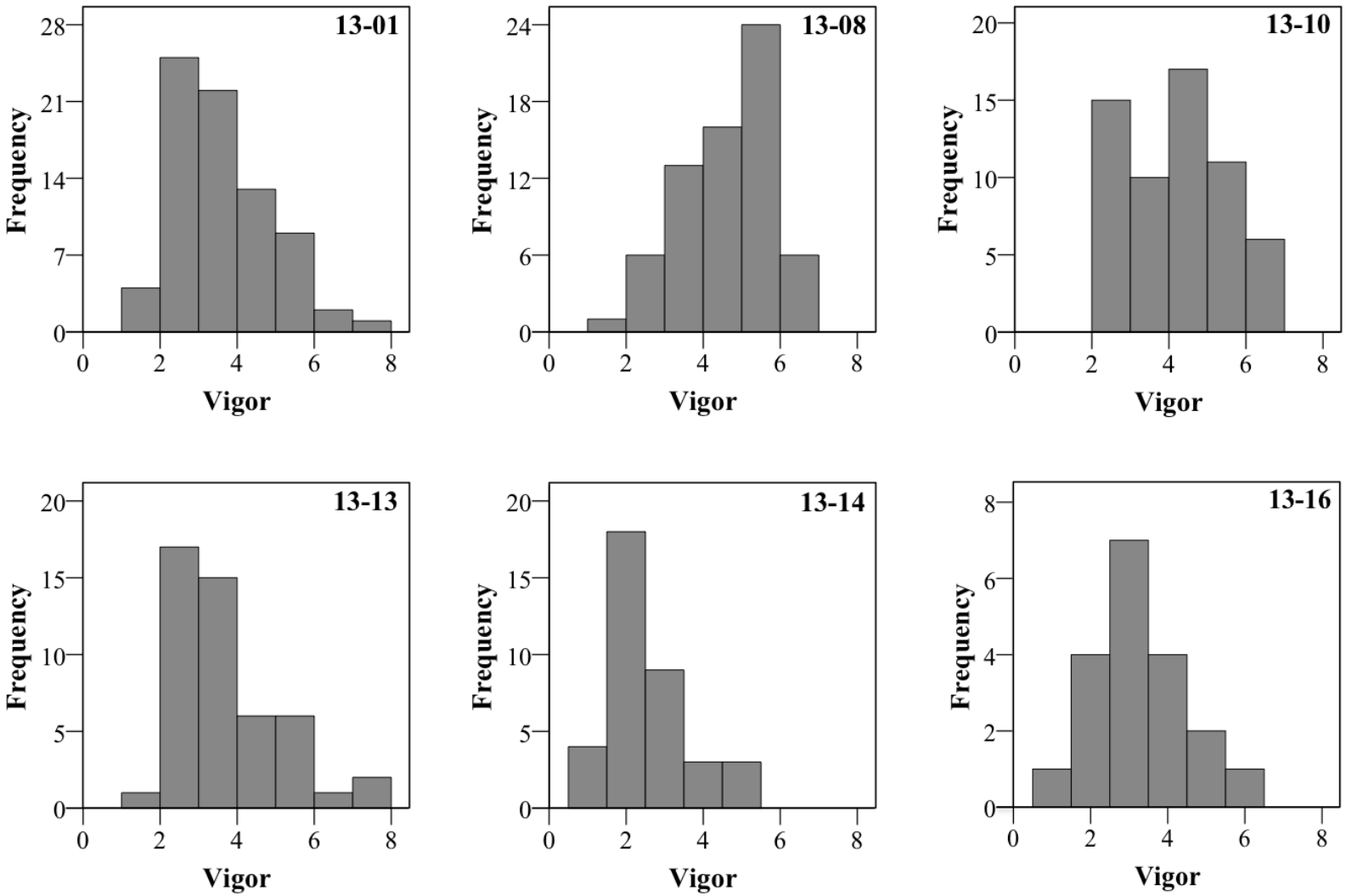

Figure 3.19. Frequency distributions of vigor rating for individuals composing six populations as described in Table 3.3. Vigor index used was a scale of 1-7, where 1 is a plant with small stature, small leaves and an unhealthy appearance not caused by disease, and 7 is very healthy, has great potential for biomass with abundant lateral branching supporting secondary branches.

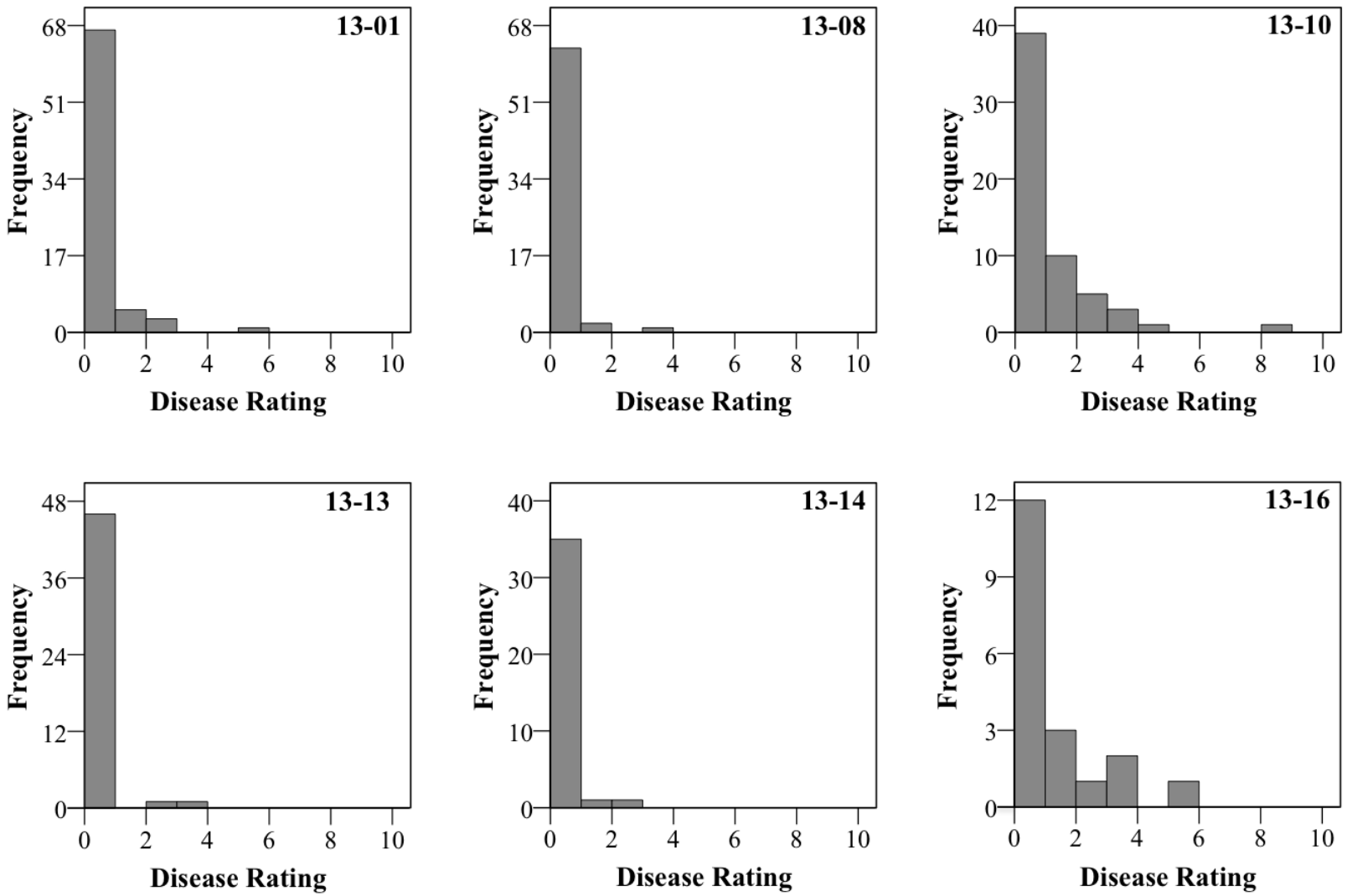

Figure 3.20. Frequency distributions of disease rating for individuals composing six populations as described in Table 3.3. Disease rating used was a scale of 1-10, where 1= 10% of plant affected, 2= 20% of plant affected, etc.

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