Phylogeny of the Genus *Arachis* and its Application to the Evolution of the Major Peanut Allergen Ara h 2

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Biological Sciences

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August 6, 2010
Blacksburg, Virginia

Keywords: *Arachis*, peanut, phylogeny, Ara h 2, evolution

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ABSTRACT

Peanuts (*A. hypogaea*) are an economically important crop, a source of food allergies and a member of the South American genus *Arachis.* The eighty species of genus *Arachis* have been divided into nine sections. The largest, section *Arachis,* has been further subdivided into three genome groups. The current intuitive understanding of the evolutionary relationships among *Arachis* is based on morphological, geographic and cytogenetic data, but a comprehensive phylogenetic study for the genus is lacking. A total of 48 species representing all nine sections were used to reconstruct a phylogeny based on sequence information from plastid *trn*T-*trn*F and nuclear ITS genomic regions. Phylogenetic analysis resolved section *Extranervosae* at the base, followed by sections *Triseminatae* and *Caulorrhizae*. Two major terminal lineages were recovered. One is comprised of sections *Erectoides, Heteranthae, Procumbentes, Rhizomatosae,* and *Trierectoides*, referred to here as group erectoides. The other is comprised of two major clades, arachis I (B genome, D genome, and aneuploid species) and arachis II (A genome species). The phylogenetic trees show that sequence data partially agrees with the relationships described in the monograph; however, some further investigation is necessary to clarify relationships within and among species of the two terminal lineages. In addition, the major allergen Ara h 2 from 12 wild species from across the genus was analyzed for mutations that could potentially produce a hypoallergenic ortholog. It was found that the evolution of the allergen mostly reflected the species phylogenies based on ITS and combined. The majority of substitutions and length variations were concentrated in the loop connecting helices H2 and H3. Section *Arachis* species tended to have larger H2-H3 loops, while those from other sections had shorter loops. The immunodominant epitopes #6 and #7, located within this loop, tended to contain mutations or were truncated among species outside of section *Arachis*. Dot immunoblots showed reduced IgE-binding to peptides representing portions of the H2-H3 loop from *A. guarantica* and *A. triseminata*. Orthologs from wild species have demonstrated that they could potentially contain variations of the allergen Ara h 2 that could be utilized to develop a safer peanut cultivar.

ACKNOWLEDGEMENTS

Dr. Khidir Hilu invited me to join his lab as an undergraduate and gave me the opportunity to continue the peanut project as a graduate student. He has provided me with the encouragement and support throughout my graduate education, for which I could not thank him enough. I also am very thankful for the guidance from my committee members, Dr. David Bevan, Dr. Liwu Li, and Dr. Jill Sible. Their advice and willingness to meet with me were a tremendous help throughout this project.

I would like to thank the former and current members of the Hilu lab, who were more than just lab mates. Dr. Michelle Barthet-Parker, you were a great inspiration and mentor when I was an undergraduate and during the beginning of my graduate education. Sunny Crawley, you've helped me troubleshoot problems in research, been my partner in crime in our adventures in pedagogy, and most of all, has been a great friend. Chelsea Black, Daniel Serrano, Muni Ali, and Rachel Maczis, your enthusiasm for the project was such a joy, and I thank so much for all the help you gave. Dr. Dietmar Quandt, thank you for the guidance on the analyses for the phylogeny chapter. Adrianna, Dipan, Stephanie, Atia, Keenan and Brittany: you all help keep things in the lab fun.

I also would like to thank my parents, Ralph and Teresa Friend, my siblings, Christina Pittman and Steven Friend, and my grandmother, Josefa Esguerra, for their never-ending love and support.

I thank Mary Wilkerson for reviewing many parts of my dissertation and keeping cookies and other desserts in the kitchen!

Last but not least, I would like to thank Aaron Elliott. You are my greatest support and source of encouragement. Thank you for reviewing the majority of my dissertation. Most of all, I thank you for patiently waiting for me as I worked on my degree.

DEDICATION

I dedicate this dissertation to my family who has supported their "forever student" in all of my academic pursuits.

I also dedicate this to my fellow Hokies, whom we lost on April 16, 2007, especially Ryan Christopher Clark and Michael Steven Pohle, Jr.

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Chapter 1: Literature Review

Introduction

The economically important peanut crop (*Arachis hypogaea* L.) is a member of the legume family Fabaceae (Figure 1.1). The peanut is the second-most widely grown seed legume in the world and a major contributor to the global production of consumable oil and protein. It is one of the top 30 crops that feed the world (Hammer et al., 2003) and is cultivated in over 100 countries with a 38.2 million metric tons global production (FAOSTAT, 2009). Not only is the peanut a good source of high temperature cooking oil, but also it is an economical source of proteins and nutrients. In the United States, peanuts are often consumed directly or in confections (Mottern, 1973), and the average American consumes approximately eight pounds of peanuts annually (Saavedra-Delgado, 1989).

The crop peanut is the most well-known member of the legume genus *Arachis*. The Krapovickas and Gregory (1994) monograph of *Arachis* recognized 69 species, all native to South America, and divided them into nine sections, predominantly based on morphological traits and geographic distribution: *Arachis*, *Caulorrhizae*, *Erectoides*, *Extranervosae*, *Heteranthae*, *Procumbentes*, *Rhizomatosae*, *Trierectoides*, and *Triseminatae*. An additional 11 species have been identified and taxonomically classified into the sections proposed in that monograph (Valls and Simpson, 2005). Section *Arachis*, the largest section, has been further subdivided into three groups of species based on genome types (A, B and D) as determined by the chromosome morphology and crossability data (Husted, 1933; Smartt et al., 1978a; Stalker, 1991). The majority of the *Arachis* species are diploids based on x=10 (Krapovickas and Gregory, 1994). Five tetraploid (2n = 4x = 40) and four aneuploid species (2n = 2x = 18) are present in the genus (Krapovickas and Gregory, 1994; Lavia, 1998; Peñaloza and Valls, 1997). Section *Arachis* contains two tetraploids, *A. hypogaea* and *A. monticola*, while the remaining

Figure 1.1 Illustration of the peanut crop, *A. hypogaea* by Franz Eugen Köhler (1887).

three tetraploid species (*A. glabrata*, *A. nitida*, and *A. pseudovillosa*) are members of section *Rhizomatosae* (Krapovickas and Gregory, 1994). The majority of the aneuploid species (A. *decora*, *A. palustris*, and *A. praecox*) are members of section *Arachis* (Lavia, 1998; Peñaloza and Valls, 1997); the remaining aneuploid species, *A. porphyrocalyx*, is a member of section *Erectoides* (Peñaloza and Valls, 2005).

Despite containing an economically important crop and numerous species, a comprehensive understanding of the phylogenetic relationship within genus *Arachis* is lacking. Krapovickas and Gregory (1994) presented an intuitive depiction of the "evolutionary and phylogenetic relationships" among the sections based on information from morphology, geographic distribution, and hybridization and pollen fertility data initially published by Gregory

and Gregory (1979). Based on these data, *Erectoides*, *Extranervosae*, *Heteranthae*, *Trierectoides*, and *Triseminatae* are considered to be sections of "older" origin, while *Arachis*, *Caulorrhizae*, *Procumbentes*, and *Rhizomatosae* are considered relatively more "recent" in origin (Krapovickas and Gregory, 1994). Since the publication of the monograph, studies on *Arachis* examining the evolutionary relationship have resulted in inconsistent scenarios regarding species relationships. The unclear and inconsistent evolutionary picture for the *Arachis* genus has been due to the use of small taxon sampling, lack of sectional representation, and insufficient markers (Halward et al., 1991; Hilu and Stalker, 1995; Hopkins et al., 1999; Lu and Pickersgill, 1993; Raina et al., 2001). Thus, the proposed species and section relationships of this genus have not been assessed well.

Here I present phylogenies that examine the species, genome, and sectional relationships within the genus *Arachis* generated using DNA sequence information from the nuclear ribosomal internal transcribed spacer (ITS) and plastid intergenic spacers and intron of transfer RNAs regions, *trn*T-*trn*L-*trn*F. The resulting phylogenies and species groupings were compared to the sections proposed in the monograph (Krapovickas and Gregory, 1994). This approach is not without precedent as sequence data have previously been used in phylogenetic studies to clarify relationships among economically important crops and remaining species within its genus (Buckler and Holtsford, 1996; Chacón et al., 2008; Golovnina et al., 2007; Kellogg and Appels, 1995; Warwick and Sauder, 2005). A robust phylogeny will not only benefit crop breeders seeking to improve growth and production, but also could be applied to the serious, life-threatening medical issue of peanut allergy.

Food allergies, peanut allergies in particular, have become a major health issue in the past decade because of the potential to cause severe to fatal reactions (Warner, 1999). Allergies to

peanuts and tree nuts affect approximately 1.3% of adults in America (Sicherer et al., 2003). In children, the prevalence of peanut allergies has almost doubled in the past decade (Grundy et al., 2002). Possible explanation of the increased prevalence includes earlier exposure to peanut (*in utero* or through breast milk) or through the growing number of foods that have small amounts of nuts (Hourihane et al., 1996; Sampson, 1996). Another possible explanation for the rise in food allergies is the "hygiene hypothesis," which suggests that the lack of exposure to infectious agents has caused the incidence of food allergies to increase (Strachan, 1989). The more severe reaction to food allergy is anaphylaxis, which Bock et al. (2001) reported to cause 150 deaths in the US annually. Of these, 80% are due to accidental consumption of peanuts or tree nuts (Bock et al., 2001).

The sources of peanut allergies have been identified as ten seed storage proteins found in the cotyledons of the seeds, referred to as Ara h 1-11 (Asero et al., 2002; Burks et al., 1992; Burks et al., 1991; Kleber-Janke et al., 1999; Krause et al., 2009; Pons et al., 2002; Rabjohn et al., 1999). The allergens Ara h 3 and Ara h 4 were found to be isoforms of the same protein (Boldt et al., 2005). Of the ten allergens, Ara h 2 (conglutin seed storage protein) is considered to be the most important allergen due to its ability to stimulate an allergic reaction at very low doses (Koppelman et al., 2004). Interestingly, this potent allergen exists in two forms in the cotyledon (Chatel et al., 2003). The allergen has been studied mainly in the peanut crop; however, Ramos (2006) has looked at the Ara h 2 allergen in wild species proposed as the progenitors of the crop using Southern blotting, sequence comparison, genomic *in situ* hybridization (GISH) and fluorescence *in situ* hybridization (FISH). Each of the two isoforms of the Ara h 2 proteins was contributed by A and B genome progenitors of the tetraploid peanut crop, *A. duranensis* and *A. ipaensis*, respectively.

In this dissertation, I also examined the molecular changes in the allergen Ara h 2 in fifteen wild *Arachis* species. The focus was on the changes in a loop region that contains epitopes consistently recognized by peanut sensitive persons. Bioinformatics methods were utilized to assess the changes and their potential effects on the protein structure. Homology modeling was used to predict the influence mutations and insertions/deletions to the amino acid sequence had on the tertiary structure. I predicted potential antibody binding epitopes using T-cell epitope programs to determine if the Ara h 2 orthologs were less likely to be allergenic. Mutations found in the loop region with immunodominant epitopes were evaluated using dot immunoblotting. Differences in antibody binding affinity for the loop regions were assessed through the use of synthetic peptides probed with Ara h 2 specific antibodies generated in chicken or sera from peanut-sensitive persons.

In addition to looking at the variation in the allergen Ara h 2 in wild *Arachis* species, I have also compared the species grouping for the gene and phylogeny for genus *Arachis*. The genus phylogeny will be used as a scaffold on which the evolution of the allergen gene will be mapped. The evolution of the allergen Ara h 2 orthologs in the peanut genus serves as a case study that can set the stage for future studies examining allergenic properties of the protein in the wild species. Genes found to be hypoallergenic can potentially be transferred to the crop.

Genus Arachis

Taxonomy and Geographic Distribution

The genus *Arachis* is a member of the family Fabaceae, subfamily Papilionoideae, tribe *Dalbergieae* (Wojciechowski et al., 2004). Pohill (1981) initially placed *Arachis* in the tribe *Aeschynomeneae* despite the lack of lamented pods. Based on *mat*K/*trn*K sequence data, Lavin et al. (2001) resolved *Arachis* in the tribe *Dalbergieae* within the *Pterocarpus* clade.

Linnaeus described the crop species *Arachis hypogaea* in 1753. The first wild species of *Arachis* were not described until almost ninety years later (Bentham, 1841). Taxonomic assessments of the genus were completed in the early to mid-twentieth century (Chevalier, 1933; Chevalier, 1934, 1936; Hoehne, 1940), but these evaluations did not categorize species consistently. Each taxonomist had his own classification and, thus, strong incongruence existed. In the early 1970s and early 1990s, researchers attempted to clarify the taxonomic nomenclature for sections within *Arachis* (Table 1.1; Gregory et al., 1973; Krapovickas, 1969; Singh and Simpson, 1994). However, these taxonomic treatments of the genus were not considered to be valid under the rules of the International Code of Botanical Nomenclature (Ressler, 1980). The latest treatment of the *Arachis* genus by Krapovickas and Gregory (1994) provided detailed descriptions of species and compiled geographic, morphological, and crossability data. This classification recognized sixty-nine species in the genus and divided them into nine sections (Table 1.1). Eleven species were recognized by Simpson and Valls (2005) based on an examination of additional taxa samples collected since the publication of the monograph. Simpson and Valls (2005) identified three new species and elevated eight samples to species status.

Table 1.1 The classification of *Arachis* sections with genomes of each section are designated within the parentheses.

Gregory et al., 1979	Singh and Simpson, 1994	Krapovickas and Gregory, 1994
Ambrinervosae (AM)	*Ambrinervosae* (AM)	*Arachis* (A_1, A_2, A_3)
Arachis (A_1, A_2, A_3)	*Arachis*(A_1, A_2, A_3)	*Caulorrhizae* (C)
Caulorrhizae (C)	*Caulorrhizae* (C)	*Erectoides* (E_2)
Erectoides (E_1, E_2, E_3)	*Erectoides* (E_1, E_2)	*Extranervosae* (Ex)
Extranervosae (Ex)	*Extranervosae* (Ex)	*Heteranthae* (AM)
Rhizomatosae (R_1, R_2)	*Procumbentes* (E_3)	*Procumbentes*(E_3)
Triseminalae (T)	*Rhizomatosae* (R_1, R_2)	*Rhizomatosae* (R_1, R_2)
	Triseminalae (T)	*Triseminatae* (T)
		Trierectoides (E_1)

Geographically, the *Arachis* species can be found east of the Andes Mountains and south of the Amazon River (Figure 1.1), mainly in Argentina, Bolivia, Brazil, Paraguay, and Uruguay (Krapovickas and Gregory, 1994). The distribution of the species throughout these countries follows the flow of the rivers, which are hypothesized to be the main route of seed distribution for this genus. The areas around the eastern boarder of Bolivia and western Brazil are believed to be the areas in which the majority of species diversity lies (Jarvis et al., 2003). Gregory et al. (1980) cited central Brazil as the center of origin for *Arachis* and northern Argentina or southern Bolivia as the center of origin of the crop peanut.

Arachis hypogaea gene pools

The peanut crop is known to have a narrow genetic background and thus improvement through the use of germplasm from wild *Arachis* species could be used to improve the crop's ability to

resist disease and drought (Kochert et al., 1996). Gene pools provide plant breeders an informal view of the relationships between the crop and its wild relatives. Harlan (1992) provided broad descriptions of gene pools to indicate the genetic relationship between a crop and its related wild species. The primary gene pool (GP-1) consists of the wild and cultivated varieties that are defined to be part of the same species (Figure 1.1). When crossing members of GP-1, fertile hybrids produced appear to have good chromosome paring and, thus, gene integration would be straightforward. For the cultivated peanut crop, wild and domesticated varieties of *A. hypogaea* are considered to be part of the primary gene pool (Rao and Murty, 1994). Another species considered to be part of the GP-1 for the crop is *A. monticola*. The other allotetraploid species of section *Arachis* can produce fertile hybrids with the crop (Stalker, 1990; Wynne and Halward, 1989). The second gene pool (GP-2) as defined by Harlan (1992) consists of species that can produce hybrids with the crop; albeit, these hybrids are often sterile, may not reach maturity, or are difficult to propagate. Based on this definition of GP-2 species, *A. monticola* could be included in the GP-1 for *A. hypogaea*. In accordance with Harlan (1992), the GP-2 is comprised of diploid species taxonomically classified within section *Arachis* as hybridization between the crop and these species rarely produces viable progeny (Wynne and Halward, 1989). The tertiary gene pool (GP-3) consists of species that can be crossed with the crop and produce hybrids that are sterile or abort during development (Harlan, 1992). Species belonging to the other sections of *Arachis* are considered to be members of the GP-3 as these species are unable to produce fertile, viable hybrids when crossed with *A. hypogaea* (Gregory and Gregory, 1979; Krapovickas and Gregory, 1994).

Figure 1.2 Geographic distribution of *Arachis* species across South America as described by Krapovickas and Gregory (1994) shown in gray shaded area. Countries where *Arachis* species are found are indicated.

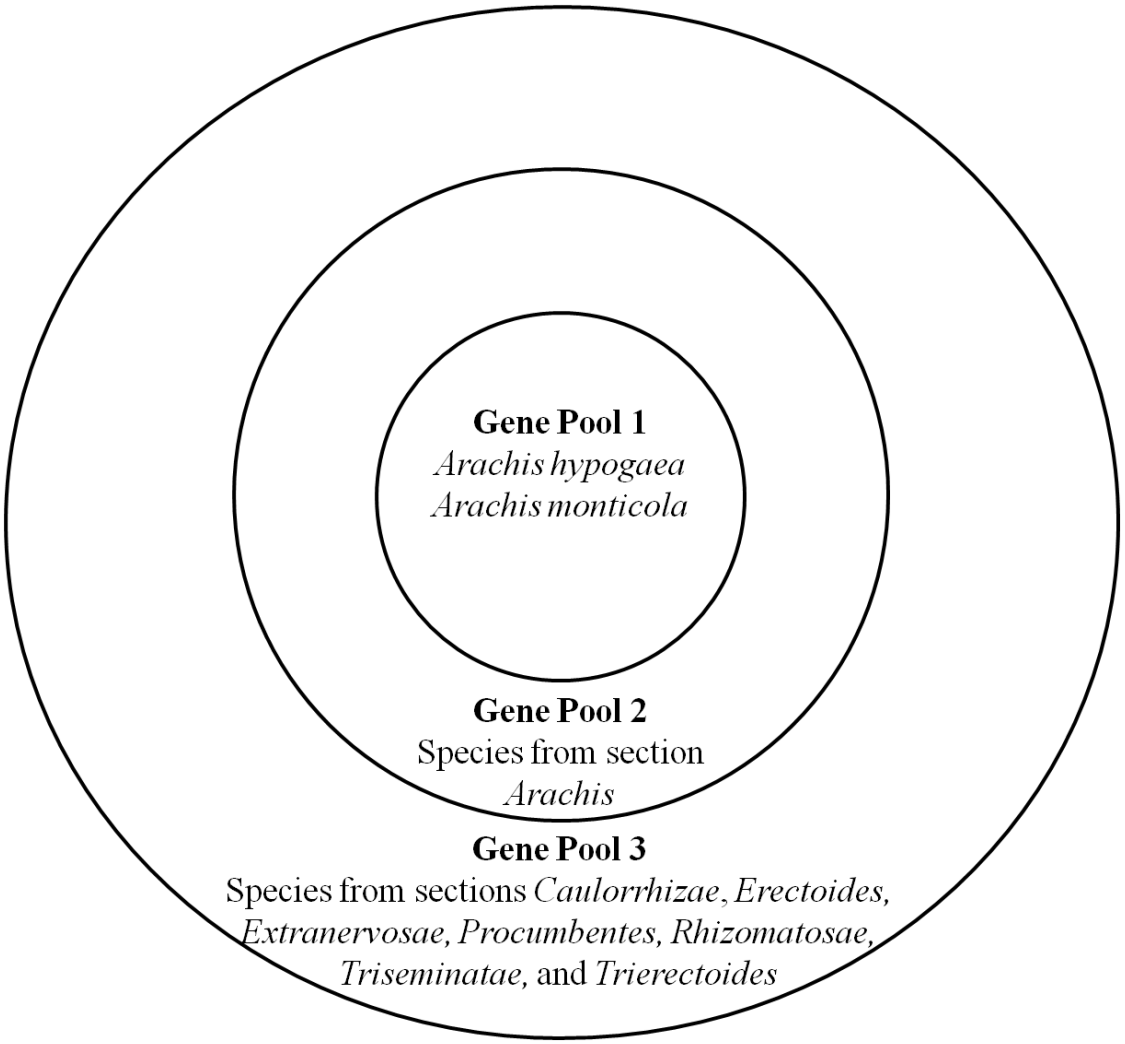

Figure 1.3 Gene pools for the peanut crop *Arachis hypogaea*.

Evolutionary relationships among the sections of *Arachis*

The majority of studies examining species of the peanut genus *Arachis* generally focus on section *Arachis*, which contains the crop *A. hypogaea*. Very few studies have investigated the other sections, and none in great detail.

Gregory and Gregory (1979) conducted one of the first studies examining intrasectional and intersectional relationships in *Arachis* based on crossability and pollen fertility data. In this study, ninety-one accessions of nineteen species of *Arachis* and ten varieties of *A. hypogaea* were included to examine the cross-compatibility relationship. Pollen fertility within sections ranged from 0.2% (section *Extranervosae*) to 86.8% (section *Caulorrhizae*). The average pollen fertility from hybrids produced from intersectional crosses was low, 1.9%. Species from section *Erectoides* had more success at generating intersectional hybrids than any other section. Also, Gregory and Gregory (1979) concluded that species of section *Erectoides* retained shared elements with sections *Arachis*, *Caulorrhizae*, *Heteranthae*, and the tetraploid species of section *Rhizomatosae*. At the time this study was conducted, sections *Procumbentes* and *Trierectoides* were included in section *Erectoides*. Based on these results Gregory and Gregory (1979) suggested that section *Erectoides* shared a common ancestor with the sections that produced hybrids. Species of section *Triseminalae*, presently recognized as section *Triseminatae* (*A. triseminata*), and the diploid species for section *Rhizomatosae* (*A. burkartii)* did not produce hybrids with any other sections. While the ability to obtain intersectional hybrids is an important indicator of sectional relationships, genetic barriers developed from the autogamous reproductive system could mask relationships among sections (Krapovickas and Gregory, 1994). Krapovickas and Gregory (1994) combined data on geographic distribution, morphology with the crossability

and pollen sterility data from the Gregory and Gregory (1979) study and proposed an intuitive prediction of species relationship among sections within genus *Arachis* (Figure 1.3).

Species and sectional relationships among *Arachis* have also been evaluated based on molecular information (Barkley et al., 2007; Creste et al., 2005; Galgaro et al., 1998; Galgaro et al., 1997; Gimenes et al., 2002a; Gimenes et al., 2000; Santos et al., 2003). Galgaro et al. (1998) used restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNAs (RAPD) markers to study the relationships among 13 species from sections *Arachis*, *Caulorrhizae*, *Extranervosae, Heteranthae*, and *Triseminatae*. The data were analyzed phenetically using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Phenetic methods predict species relationships based on the presence or absence of data and do not consider the evolutionary history. The phenograms generated using RFLP and RAPD data resulted in three major clusters. Two of the clusters were homogeneous: one cluster comprised of species from section *Extranervosae*, and the second cluster was made up of the four accessions of *A. hypogaea*. The third cluster was a heterogeneous cluster that included species of sections *Caulorrhizae, Triseminatae,* and *Heteranthae*. UPGMA phenograms based on RAPD or RFLP data used in the Galgaro et al. (1998) were not in agreement on the placement of *A. dardani* of section *Heteranthae* and placed the validity of the section in question.

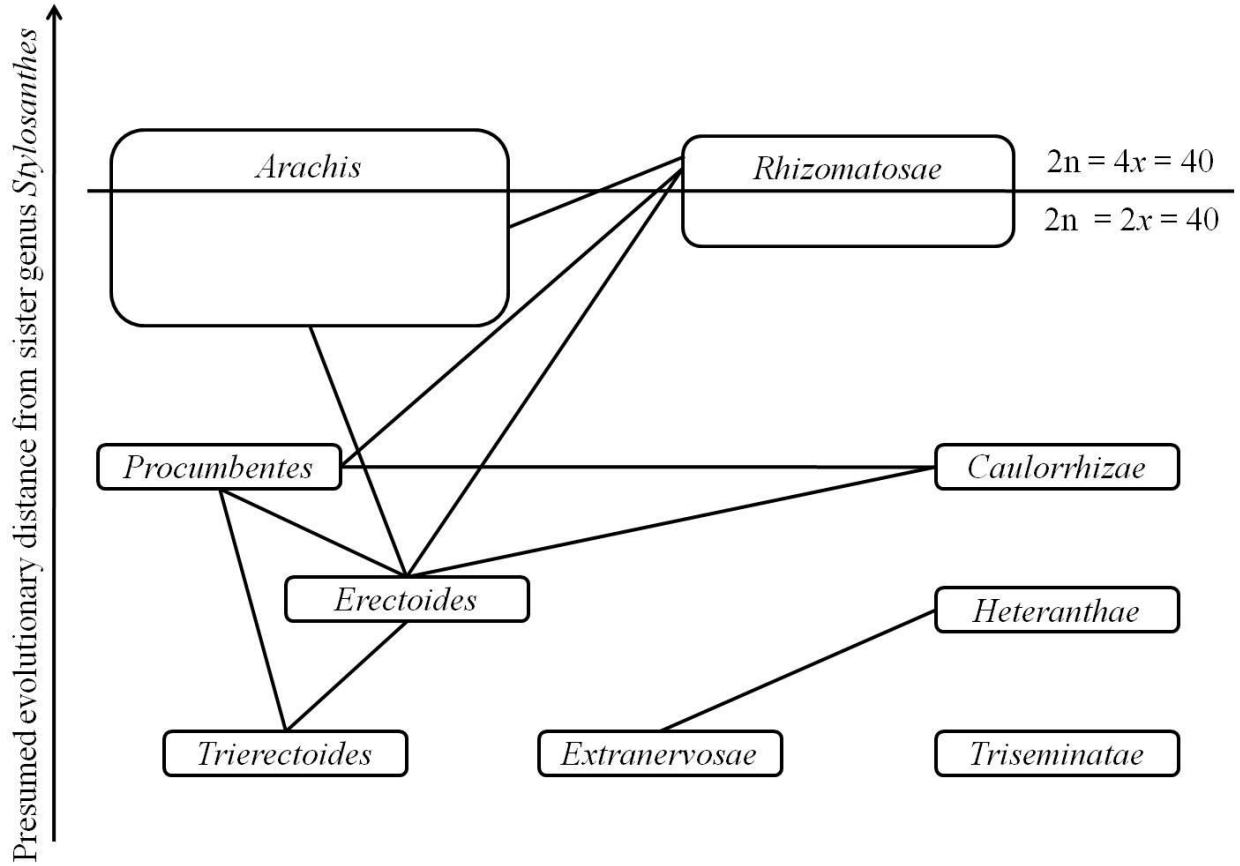

Figure 1.4 Sectional relationships within genus *Arachis* presented in the monograph by Krapovickas and Gregory, (1994). Lines connecting sections were based on the hybridization data by Gregory and Gregory (1979).

Santos et al. (2003) used RAPD data analyzed using phenetic methods to examine relationships among sections *Arachis*, *Erectoides*, *Procumbentes*, *Rhizomatosae*, and *Trierectoides*. The accessions formed two clusters in the dendrogram; one consisted of species from section *Arachis*, while the other cluster contained species from the remaining sections divided into two subclusters. They suggested that sections *Procumbentes* and *Rhizomatosae* are more closely related to sections *Erectoides* and *Trierectoides* than to section *Arachis*. Sections *Erectoides*, *Procumbentes*, and *Trierectoides* depict a genetic affinity that was previously recognized by Gregory and Gregory (1979).

In another UPGMA phenetic analysis, Gimenes et al. (2002b) used Amplified Fragment Length Polymorphism (AFLP) markers to assess relationships among twenty species from sections *Arachis*, *Caulorrhizae*, *Erectoides*, *Extranervosae*, *Heteranthae*, *Procumbentes*, and *Rhizomatosae*. Three major clusters were also recovered in their phenogram. Section *Rhizomatosae* species did not group together as expected. The diploid member of this section, *A. burkartii*, was grouped with species from section *Caulorrhizae*, while the tetraploid species, *A. glabrata*, grouped with species from section *Erectoides*. This study suggests that the diploid and tetraploid species of section *Rhizomatosae* are not closely related, and thus, the validity of this section comes into question.

Hoshino et al. (2006) was the first to represent all sections of *Arachis* using microsatellites and analyzing the data with UPGMA. The majority of the species grouped in their respective sections as defined by Krapovickas and Gregory (1994). However, the validity of section *Heteranthae* was once again questioned as members of section *Heteranthae* failed to group together, which was previously observed in the Galgaro et al. (1998) study.

Of the studies that examined the intersectional relationships within genus *Arachis*, very few had commented on its evolutionary history (Gregory and Gregory, 1979; Krapovickas and Gregory, 1994). Gregory and Gregory (1979) concluded that the "most ancient of species" in *Arachis* were from sections *Extranervosae*, *Erectoides* (E1), and *Rhizomatosae* (R_1) based on data from cross-compatibility. In the monograph by Krapovickas and Gregory (1994), an institutive depiction of the evolutionary and phylogenetic relationships among the *Arachis* sections was provided. The E_1 genome group was raised to sectional classification, section *Trierectoides*, and was considered to be the "most primitive" of the genus due to its high degree of genetic isolation and morphological characters that are found only in this section. The phylogenetic relationship of the *Arachis* genus remains unclear since the studies to date examining this genus looked at selected sections or used few representative species and none used a phylogenetic approach (Galgaro et al., 1998; Gimenes et al., 2002a). A study examining relationship of the entire genus and with adequate sampling is needed.

Genomes of Section *Arachis*

Section *Arachis* is the largest in the genus, containing thirty-one species (Krapovickas and Gregory, 1994). The majority of these species are diploid (2n=2x=20). This section also includes two tetraploid species (2n=4x=40), *A. hypogaea* and *A. monticola*, and three aneuploid species (2n =2*x* =18), *A. decora*, *A. palustris*, and *A. praecox* (Lavia, 1998; Peñaloza and Valls, 2005). Species in section *Arachis* have been further subdivided into three genome groups, A, B, and D, based on cytological data, such as chromosome pairing and hybrid fertility (Husted, 1933; Smartt et al., 1978a; Stalker, 1991).

Husted (1933; 1936) was the first to describe two distinct chromosomes (A and B) in *A. hypogaea* based on observations of chromosome pairing during meiosis. The A chromosomes were noticeably smaller than the rest, while the B chromosome pair contains a second constriction with satellites. The difference between the two unique chromosomes indicated that *A. hypogaea* contained two genomes (Smartt, 1965). Species of section *Arachis* in which the small A chromosome is present have been designated as A genome species, while those lacking the A chromosome are B genome species (Smartt et al., 1978). The third genome in section *Arachis*, D genome, is found only in *A. glandulifera* (Stalker, 1991). The D genome lacks the chromosomes distinct to the A and B genomes, and has a karyotype that is only seen in accessions of *A. glandulifera*.

The majority of species of section *Arachis* contain the A genome. *Arachis batizocoi* was the first species of section *Arachis* described to contain the B genome (Smartt et al., 1978a). Artificial hybrids among species of section *Arachis* were fertile with the exception of hybrids from crosses with *A. batizocoi* (Smartt et al., 1978a). Hybrids produced using species with the A genome had moderate to high pollen stability, while hybrids produced with *A. batizocoi* (B genome) had no pollen stability. While stable pollen production was not present in hybrids with *A. batizocoi*, the ability to produce a hybrid with species from section *Arachis* suggests that the evolutionary divergence of the A and B genomes occurred recently. Species with this genome designation appear to be fairly diverse. While the genome of *A. ipaensis* was not confirmed cytogenetically until 1994 by Fernández and Krapovickas, it has an RFLP banding pattern that is similar to the B genome species *A. batizocoi,* when compared to the A genome species (Kochert et al., 1991). Seijo et al. (2004) examined banding patterns in section *Arachis* species using fluorescent *in situ* hybridization (FISH) and noted that *A. batizocoi* has a distinct profile when

compared to profiles from other B genome species; *Arachis ipaensis* and *A. williamsii*, can be clustered into their own subgroup. Based on information from FISH data, Burow et al. (2009) suggested that the B genome species in section *Arachis* should be divided into two groups, the *batizocoi* group and the *ipaensis* group. Section *Arachis* species *A. cruziana* was grouped with *A. batizocoi*, while *A. ipaensis* formed a group with *A. magna*. The separate grouping of the B genome species suggests that the B genome group might need to be split into two groups. However, more data will be needed to taxonomically divide the B genome species into the *batizocoi* and *ipaensis* groups.

Morphologically, the A and B genome species are similar based on leaf size, pod shape and length, flower shape and width, and meristem lengths (Stalker, 1990). In a phenogram depicting species relationship based on morphology alone, *A. ipaensis* (B genome) was considered to be most morphologically distinct from other species of section *Arachis* (Stalker, 1991). Species that had the unique D genome karyotype did not cluster with the rest of the groups from section *Arachis*. Within a morphology-based dendrogram, species with the A and B genomes were mixed rather than forming separate clusters. This means that the A and B genomes are morphologically closer to each other than either of them to the D genome.

In addition to the diploid species containing the A, B, or D genomes, section *Arachis* also contains aneuploid species. The aneuploid species have a base chromosome number of x=9 (2n=18) (Lavia, 1998). These species lack the small chromosomes characteristic of the A genome species. The basic chromosome number of x=9 of these species could have been derived from an *Arachis* species with x=10. If this is the case, then the aneuploid species are of a more recent species evolution.

Our recent study based on plastid sequence information found that the B and D genomes were more closely related to each other than to the A genome (Tallury et al., 2005). The aneuploid species, *A. palustris* and *A. praecox*, did not group with the A genome group, nor to the B and D genome group. This suggests that they could represent an intermediate between the A genome and the other two genomes. Further study examining the relationship among the section *Arachis* genomes and aneuploids would contribute in a better understanding of these relationships.

Origin of the tetraploid crop peanut, *A. hypogaea*

Only a handful of species in genus *Arachis* are tetraploids; the most noted of these is the cultivated crop, *A. hypogaea*. The crop is an allotetraploid because it a hybrid containing both the A and B genomes present in section *Arachis* species (Husted, 1933; Stebbins, 1957). The origin of the peanut crop could have occurred by amphidiploidization of a hybrid of A and B genome wild species (Singh, 1988; Singh and Moss, 1984). Section *Arachis* species have long been considered to be potential progenitors since crosses with these species and with the tetraploid crop have resulted in successful hybrids (Krapovickas and Gregory, 1994; Smartt and Stalker, 1982; Stalker, 1990; Stalker and Moss, 1987). Understanding of species relationships in section *Arachis* is important because these are considered to be genetic resources for the crop (Smartt et al., 1978b). These are important because *A. hypogaea* has a narrow genetic background.

Various diploid species in section *Arachis* have been suggested to be the progenitors of the crop. Initially based on cytogenetic evidence, potential progenitors with the A genome included *A. cardenasii*, *A. duranensis*, and *A. villosa* (Seetharam et al., 1973; Smartt et al., 1978a; Varisai Muhammad, 1973). Seed storage protein profiles have also provided evidence, in addition to cytogenetics, for *A. cardenasii* as a potential progenitor (Krishna and Mitra, 1988).

In addition to the cytogenetic evidence, FISH studies have provided support for *A. villosa* as a potential progenitor (Raina and Mukai, 1999; Raina et al., 2001). Recently, Milla et al. (2005) suggested *A. helodes* and *A. simpsonii* as potential A genome donors based on AFLP data. However, the majority of studies examining potential progenitors have focused on *A. duranensis* as the A genome progenitor of *A. hypogaea* based on data from seed storage protein profiles, isozyme (Lu and Pickersgill, 1993), RFLP (Kochert et al., 1991; Kochert et al., 1996), RAPD (Hilu and Stalker, 1995), DNA sequence information (Jung et al., 2003), and FISH (Seijo et al., 2004).

For the B genome, *A. batizocoi* had long been assumed as the B genome donor for the crop (Krapovickas, 1969). However, this assumption was due to the lack of other identified B genome species. Based on evidence from RFLP, Kochert et al. (1991) found *A. batizocoi* not to be as closely related to *A. hypogaea* as previously thought. Other studies have also supported the exclusion of *A. batizocoi* as the B genome donor based on RAPD (Hilu and Stalker, 1995), RFLP (Burow et al., 2009; Kochert et al., 1996), isozymes (Lu and Pickersgill, 1993), and FISH (Seijo et al., 2004). Kochert et al. (1991) was the first to suggest *A. ipaensis*, a recently identified species, to be the B genome donor. At the time, *A. ipaensis* was considered to be an A genome species. However, Kochert et al. (1991) was the first to show that *A. ipaensis* was a B genome species using RFLP markers. This finding was later confirmed by Fernández and Krapovickas (1994) based on karyotpying and Tallury et al. (2005) using plastid DNA sequences.

In the case of the other tetraploid classified species in section *Arachis*, *A. monticola* has been proposed to be a wild progenitor of the crop (Smartt et al., 1978a). The two allotetraploid species are very similar morphologically, hybridize successfully producing fertile hybrids, and have overlapping areas of geographic distribution (Simpson et al., 2001). Some

researchers believe that *A. monticola* is a weedy species that evolved from the cultivated crop (Gregory and Gregory, 1976; Halward et al., 1991). Data from RAPD, RFLP, and AFLP have shown that markers from the two allotetraploid species were nearly identical (Halward et al., 1991; Hilu and Stalker, 1995; Kochert et al., 1991; Milla et al., 2005), which does not support the hypothesis that *A. monticola* was a progenitor to *A. hypogaea,* as the two tetraploids could be considered a single species genetically.

The consensus is now that *A. duranensis* and *A. ipaensis* are the A and B genome donors, respectively, for the crop *A. hypogaea* (Jung et al., 2003; Kochert et al., 1991; Kochert et al., 1996; Seijo et al., 2004). Burow et al. (2009) attempted to reconstitute the RFLP markers identified in *A. hypogaea*. Of the crosses attempted, the combinations that gave the greatest match to the crop were ones that crossed *A. duranensis* and *A. ipaensis*. However, the best hybrid generated only 51% of the RFLP markers found in *A. hypogaea*. Other hybrids that were able to produce a smaller portion of the RFLP bands similar to the crop were generated with *A. ipaensis* as one of the parent species. The *A. duranensis* x *A. ipaensis* hybrids only produced about half of the RFLP markers as compared to the crop. The lack of bands could be explained by the rapid genomic restructuring after a polyploidy speciation event has occurred; however, Burow et al. (2009) also suggested that additional *Arachis* species that contained genomes more similar to the crop could still be identified in the future.

Identification of additional species as potential progenitors for *A. hypogaea* would provide a better understanding of its origin (Burow et al., 2009). In addition, a clearer understanding of tetraploid evolution would allow for the production of hybrids that are able to cross with the crop and the introduction of alleles that could benefit farmers and consumers of peanuts.

Peanut allergy

Peanut is one of the eight foods that cause 90% of the immunoglobulin E (IgE)-associated food allergies (Lehrere et al., 2002). Consequently, these foods have been termed the "Big Eight." The other foods that make up the "Big Eight" tree nuts, cow's milk, soy, wheat, hen's egg, fish, and crustaceans. Branum and Lukas (2008) concluded that food allergies as a whole increased by 18% between 1997 and 2007. The cause of the rise in food allergies, particularly those to peanut, has not been well studied (Burks, 2008). Currently, 4% of children are predicted to have food allergies (Sampson, 1999; Sicherer et al., 2003). Peanut allergies are among the most severe food allergies because they have the ability to induce anaphylaxis. In 2002, allergies to peanuts affected approximately 0.8% of children and 0.6% of adults in the U.S. (Sicherer et al., 2003). The percent of children with allergies to peanut doubled from 0.4% in 1997 to 0.8% in 2002 (Sicherer et al., 2003). Accidental consumption of peanuts is considered to be the cause of two hundred anaphylaxis deaths annually (Bock et al., 2001). Other than emergency treatments with antihistamine and epinephrine pins, preventative treatments for allergies include avoidance, pharmacological treatment, and allergy-specific immunotherapy. For peanut allergies, the only preventative treatment currently available for peanut allergies is avoidance (Burks, 2008).

Peanut allergens

In the peanut cotyledon, ten proteins have been identified as allergens (Asero et al., 2002; Burks et al., 1992; Burks et al., 1991; Kleber-Janke et al., 1999; Krause et al., 2009; Pons et al., 2002; Rabjohn et al., 1999). Like most plant food allergens, the majority of the peanut allergens fall into one of a few protein families or superfamilies (Table 1.2).

Cupin superfamily is considered to be a large superfamily of proteins that are very diverse functionally (Dunwell et al., 2004). The superfamily is named for the β-barrel core domain and can be divided into two groups; the single-domain cupins (monocupins) and two-domain cupins (bicupins). The 7/8S and 11S seed storage proteins that have also been classified as allergens are considered to be one of the largest groups within bicupins. The major peanut protein Ara h 1 is a 7S globulin while the allergen Ara h 3/4 is an 11S globulin (Mills et al., 2004).

The prolamin superfamily includes proteins that are both water- and alcohol-soluble and tend to be proline- and glutamine-rich (Breiteneder and Mills, 2005). Members of this protein superfamily tend to be low in molecular weight, with the secondary structure being primarily α-helical in nature and the tertiary structure stabilized by six to eight conserved cysteines that form three or four disulfide bonds within the protein. Despite the conserved nature of the structure and cystine motifs, members of this superfamily are very diverse, and have a variety of functions. These include 2S seed storage albumins, non-specific lipid transfer proteins, and protease inhibitors. In peanut, the major allergens Ara h 2 and Ara h 6, and minor allergen Ara h 7 are members of this protein superfamily. The recently identified Ara h 9, considered to be a minor allergen has been classified as a non-specific lipid transfer protein and appears to be an important allergen in Mediterranean regions (Krause et al., 2009).

The profilin family, another protein family whose members include food allergens, consists of cytosolic proteins of small molecular weight (12-15 kDa). They generally are involved with cellular processes such as signaling cytokinesis and cellular movement (Witke, 2004). Allergens that are from this protein are highly allergenic and are known to elicit allergic response from 10-20% patients who are allergic to pollen (Valenta et al., 1992). In peanuts, the

only identified allergen classified as part of the profilin superfamily is Ara h 5 (Kleber-Janke et al., 1999).

The allergen Ara h 8 has similarities to the major birch pollen allergen Bet v1 (Mittag et al., 2004), which belongs to the subfamily of pathogenesis-related protein PR10 (Raudauer and Breiteneder, 2007). Like other Bet v 1-like allergens, Ara h 8 was not stable when heated or digested with gastric juices (Mittag et al., 2004), which could explain the low association of peanut IgE to this allergen.

Peanut allergens Ara h 1-9 are members of the protein superfamilies typical for plant food allergens (Table 1.2). However, the recently-recognized Ara h 10 and Ara h 11 have been classified in a protein family more noted for the formation of oil bodies (Pons et al., 2002). These allergens are hypothesized to contribute to the cross-reactivity observed between peanut and soybean. Further research needs to be conducted to fully understand the role of Ara h 10 and 11 in peanut allergies (Pons et al., 2002).

Table 1.2 Identified peanut allergens, their function and protein classification.

Allergen	Common Name	Family/Superfamily	Function	Size (kDa)	Identified
Ara h 1	Arachin	Cupin	7S Vicilin-like Globulins, seed storage	71	Burks et al.,1991
Ara h 2	2S albumin/ Conglutin	Prolamin	2S seed storage, trypsin inhibitor	17	Burks et al., 1992
Ara h 3/4	11S Globulin	Cupin	11 S Globulin seed storage	60/53	Kleber-Janke et al.,1999
Ara h 5	Profilin	Profilin	Actin-binding protein	14	Kleber-Janke et al., 1999
Ara h 6	2S albumin, Conglutin	Prolamin	2S albumin	15	Kleber-Janke et al.,1999
Ara h 7**	Conglutin	Prolamin	Conglutin	--	Kleber-Janke et al., 1999
Ara h 8**	Bet v-1 homolog	PR-10	Pathogen-resistance protein	--	Mittag et al., 2004
Ara h 9	Lipid Transfer Protein	Prolamin	Lipid Transfer Protein	9.8	Krause et al., 2009
Ara h 10	Oleosin		Oil-body formation	16	Pons et al., 2002
Ara h 11	Oleosin		Oil-body formation	14	Pons et al., 2002

** Protein information, including molecular weight, currently not known.

Conglutin Allergen: Ara h 2

The conglutin protein Ara h 2 was identified as a peanut allergen using sera from patients who were known to be allergic to peanuts (Burks et al., 1992). Ara h 2 was recognized to be present as a doublet consisting of a 16 kDa and 18 kDa isoforms. The pI of the allergen was determined to be 5.2 using a two-dimensional gel. Two isoforms of Ara h 2 have been identified in peanut cotyledons; Ara h 2.01 and Ara h 2.02 (Chatel et al., 2003). The larger isoform, Ara h 2.02, has a twelve amino acid insertion, which contains an additional DPYSPS IgE-binding epitope. Chatel et al. (2003) proposed the additional DPYSPS motif could make Ara h 2.02 a more potent isoform than Ara h 2.01, although this has yet to be confirmed. Stanley et al. (1997) used synthetic peptides corresponding to the amino acid sequence of Ara h 2 and deduced the 10 IgE binding epitopes (Table 1.3). Sera from ten peanut-sensitive patients consistently recognized three epitopes, 3, 6, and 7, which were determined to be immunodominant. Alanine mutations to certain residues within these epitopes reduced or eliminated IgE-binding (King et al., 2005). Examining Ara h 2 orthologs in proposed progenitors, Ramos et al. (2008) identified an accession of *A. duranensis* that contained a single nucleotide polymorphism (SNP), which caused an amino acid change within the same class, S73T. The amino acid mutation resulted in a 56-99% reduction in IgE-binding when probed by individual sera and quantified by densitometry.

Sequence analysis of a peanut genomic library revealed a 624 base pair open reading frame (ORF) for *Ara h 2* gene (Viquez et al., 2001). The gene *Ara h 2* appears to lack introns based on comparison between *Ara h 2* cDNA and genomic clone sequences. The genomic sequence from the Viquez et al. (2003) study was later referred to as the *Ara h 2.01* isoform, which corresponds to some published cDNA sequences of *Ara h 2* in GenBank. Chatel et al.

(2003) reported another sequence of the *Ara h 2* gene that contained a 36 base pair (12 amino acids) insertion that was named *Ara h 2.02*, which corresponds to the larger Ara h 2 protein isoform. Based on a Southern blot comparing the *Ara h 2* isoforms, Ramos et al. (2006) concluded the progenitors, *A. duranensis* and *A. ipaensis*, contributed an isoform to the allotetraploid crop. The orthologs of *Ara h 2* from the wild progenitors migrated identically to isoforms from the cultivated peanut. *Ara h 2.01* migrated the same distance as the ortholog from *A. duranensis* (A genome) and *Ara h 2.02* migrated the same distance as the ortholog from *A. ipaensis* (B genome). Sequences of Ara h 2 orthologs from the proposed progenitors, *A. duranensis* and *A. ipaensis* were 99.7-100% identical to *Ara h 2.01* and 99.4%-99.9% identical *Ara h 2.02*.

In addition, Ramos et al. (2006) elucidated the expression pattern of the two *Ara h 2* isoforms was examined by random sequencing of cDNA clones. The expression ratios were determined by RT-PCR to be 2.7:1 A:B genome. The expression ratios were similar to another study that found the expression of two conglutin isoforms to be 2:1 A:B genome from sequencing 400 cDNA clones from *A. hypogaea* (Yan et al., 2005).

Ara h 2 has been shown to function as trypsin inhibitor (Maleki et al., 2003). The function of Ara h 2 as a trypsin inhibitor increases when the peanut undergoes certain methods of thermo-processing. Roasting increases the inhibition of trypsin 3.5 times compared to unroasted peanuts. In addition to resisting degradation by trypsin, Ara h 2 also protects the allergen Ara h 1 from that protease (Maleki et al., 2003).

Table 1.3 IgE binding epitopes present in Ara h 2 represented by single-letter amino acid code initially identified by Stanley et al. (1997). Chatel et al. (2003) identified the second isoform, Ara h 2.02, which contains an additional epitope 6. Positions of epitopes for each isoform as found in the peptide sequence alignment are noted.

Epitope	Amino Acid Sequence	Ara h 2.01 Position	Ara h 2.02 Position
1	HASARQQWEL	18-27	18-27
2	QWELQGDR	24-31	24-31
3	DRRCQSQLER	30-39	30-39
4	LRPCEQHLMQ	42-51	42-51
5	KIQRDEDS	52-58	52-58
6	RDPYSPS	62-68	62-68/81-87
7	SQDPYSPS	68-75	68-75
8	LQGRQQ	120-125	132-137
9	KRELRN	130-135	142-147
10	QRCDLDVE	146-153	158-165

The Maillard reaction, which roasts food products while adding sugars, increases the Ara h 2 binding to IgE (Maleki et al., 2000). When Ara h 2 protein was roasted in the presence of glucose, there was a 2.7-fold increase in IgE binding, while roasting Ara h 2 in the presence of xylose increased IgE binding by 5.6-fold. Recombinant Ara h 2 showed similar effects when roasted in the presence of sugars (Gruber et al., 2005). Boiling peanut does not change the binding of IgE to Ara h 2, even though immunoreactivity of allergens from boiled peanut decreased two-fold. The decreases allergenicity of whole peanut is speculated to be due to the loss of low molecular weight proteins by being transferred to water during boiling (Mondoulet et al., 2005).

Homology Modeling

The three-dimensional structure of a protein can provide information about its biological function. Tertiary structures are determined experimentally through X-ray crystallography or nuclear magnetic resonance (NMR; Sali and Kuriyan, 1999). To date, more than 60,000 protein structures are available on the Protein Data Base (PDB) server. Advances in experimental techniques have speeded the process of elucidating a protein's tertiary structure. However, experimentally determining a protein structure is still a time-consuming and costly process. Thus, the number of coding DNA sequences, and thus deduced amino acid sequences, available on databases like GenBank demonstrates that there is a large gap between protein sequence and structure information (Daga, 2010). This gap in sequence-structural knowledge can be reduced through the use of protein models. Predicted protein structures can be identified using either *de novo* (threading) methods or template-based (homology) modeling (Baker and Sali, 2001). Threading examines the sequences for predicted areas of tertiary structures that would have the

lowest energies for those structures. In comparison to homology modeling, theoretical models generated using de novo methods are not considered to be very reliable.

Hypothetical tertiary structures generated using homology modeling are based on experimentally derived template structures (Baker and Sali, 2001). This approach is possible because tertiary structures of related proteins are more evolutionarily conserved than the amino acid sequence (Marti-Renom et al., 2000). Also, structural similarity can be found in sequences that are more divergent in protein families and superfamilies. The accuracy of the model is influenced by the sequence homology between the target (the protein in which the structure will be predicted) and the template (the known structure). Predicted models that have over 50% sequence identity to the template are considered to be highly accurate, with approximately 1 Å root mean square (RMS) error for the backbone atoms (Baker and Sali, 2001). Target structures with 30-50% sequence identity to the template structures are considered to have medium accuracy, with the large majority of the model considered to be accurate with about 1.5 Å RMS errors. The majority of errors detected in models with 30-50% sequence identity to the template structure can be attributed to errors in alignment. Models built using templates with less than 30% sequence identity have low accuracy and these models tend to have larger amounts of gaps and errors within the alignment (Marti-Renom et al., 2000). Models with less than 30% sequence identity to their template are not thought to be an accurate depiction of the target's tertiary conformation.

Within a protein family, the majority of changes in the form of substitution, insertion, and deletion of residues are found within areas of the sequence that correspond to loop regions connecting secondary structures (Fiser et al., 2000). Due to the location of these changes, loops tend to determine the functional aspects of a protein. In the case of plant allergens belonging to

the prolamin superfamily, IgE-binding epitopes have been found within extended loop regions (Monsalve et al., 1993; Robotham et al., 2005). Two approaches are generally used when determining the loop structure, database and conformational searches. The former approach relies on a database of known loops that are from proteins from experimentally derived tertiary structures classified based on conserved features, such as length and presence of particular residues (Burke et al., 2000; Espadaler et al., 2006; Li et al., 1999). The database approach can be an accurate and efficient method as long as the target loop is in the same class of loops (Fiser et al., 2000). This method is limited due to the lack of known loop structures available and the length of loops being modeled. Generally, loop conformation databases search for loops less than ten residues in length. However, attempts have been made to recognize larger loops (Schlessinger et al., 2007; Vucetic et al., 2005). Conformational searches can employ a variety of search algorithms to elucidate the structure of a loop. Examples of these algorithms could include global energy minimization (Dudek et al., 1998), local energy minimization (Mattos et al., 1994), molecular dynamics simulations (Nakajima et al., 2000), and optimization-based approaches (Fiser et al., 2000).

Homology models of the Ara h 2 allergen from the peanut crop

Determination of the protein structure of Ara h 2 would yield better understanding of the allergen's interaction with IgE antibodies and how it elicits an immunological response. The structure of the peanut Ara h 2 protein was first modeled using Ric c 3, a 2S albumin from castor bean (*Ricinus communis*; Figure 1.4A), which shares approximately 37% sequence identity with Ara h 2 (Barre et al., 2005). The molecular model of Ara h 2 shows that it is a right-handed alpha helical superstructure and has been confirmed by a homology model generated from the

recently released structure of the minor allergen Ara h 6 (Figure 1.4B; (Lehmann et al., 2006). The two peanut conglutin proteins share a 63% overall sequence identity. Considering only the alpha-helical regions, the sequence identity increases to 75%. Comparing Ara h 2 to the determined structures of Ric c 3 and Ara h 6, Ara h 2 contains five alpha-helices (Barre et al., 2005; Lehmann et al., 2006). Four disulfide bridges stabilize the structure of the protein. These disulfide bridges have been associated with the allergen's ability to be digested by gastric enzymes chymotrypsin, pepsin, and trypsin (Sen et al., 2002).

Structural information would provide more insight into how the plant protein functions as an allergen and lend insight on the mechanism that allows for some proteins to cross-react with each other (Jenkins et al., 2005). Information on allergen structure could provide a clearer picture of what contributes to the classification of Ara h 2 as an allergen. The current understanding of the presence of this allergen in related wild *Arachis* species is limited. Examining other wild species for this allergen could reveal natural mutants. It would also provide information that could be used to develop therapeutic approaches to allergy treatment (Radauer et al., 2008).

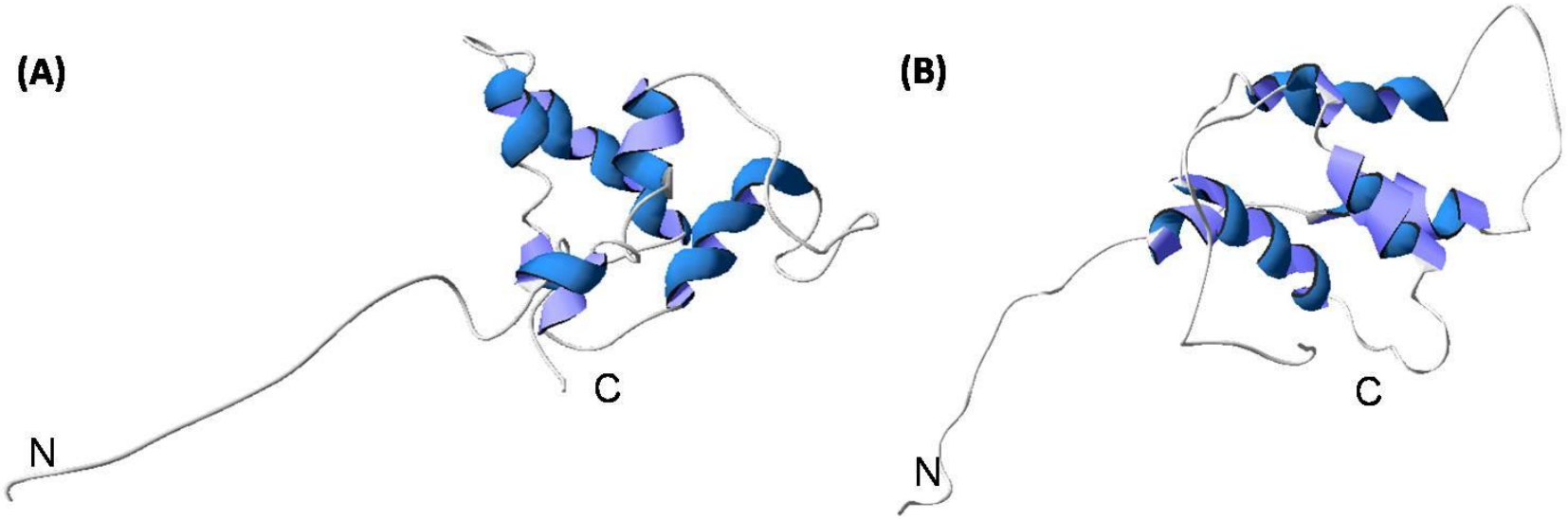

Figure 1.5 NMR-determined structures of (A) Ric C 2 (PDB ID 1PSY) from castor bean (*Ricinus communis*; Pantoja-Uceda et al. 2003and (B) Ara h 6 (PDB ID 1W2Q) from peanut (*Arachis hypogaea*; Lehmann et al., 2006), which have been used as template structures for homology models of Ara h 2 from *A. hypogaea*. Coordinates for the structures were obtained from the RCSB Protein Databank and rendered in Swiss-PdbViewer v4.0.1

Project Objectives

The peanut crop comes from a genus that contains numerous species, many of which are potential genetic resources. However, the evolutionary relationships among species, genomes, and sections of the genus *Arachis* are not well understood. The first objective in this dissertation is to present phylogenies examining relationships within genus *Arachis* based on DNA sequence information from plastid and nuclear genomic regions. This will be the first phylogenetic study examining species, genomes and sectional relationships for the whole genus since the publication of the Krapovickas and Gregory (1994) monograph. The phylogenies produced here can be used as a guide for improving the peanut crop not only from the agricultural perspective but also from medical one. The second objective is to examine molecular changes within a protein that is a major allergen, Ara h 2, from wild *Arachis* species. The species chosen were representatives of the major lineages present in the phylogenies for the genus. In this dissertation, I have examined the mutations among the Ara h 2 orthologs from *Arachis* wild species and their effects on the secondary and tertiary structure of the protein. The mutations observed in Ara h 2 orthologs were evaluated for changes in potential antibody binding particularly in a loop region that contains immunodominant IgE-binding epitopes. Differential antibody binding to orthologs with significant mutations in these IgE-binding sites was assessed using dot immunoblots. The results presented here will be the first to examine the major allergen outside of the peanut crop and its progenitors and has the potential to identify wild *Arachis* species that could be utilized for treatment of peanut allergies.

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CHAPTER 2: Species, genomes and section relationships in genus *Arachis* (Fabaceae): A molecular phylogeny

Abstract

The economically important genus *Arachis* (Fabaceae) is comprised of 80 species restricted to South America. There is only one monograph for the genus that divided it into nine sections and provided an intuitive assessment of evolutionary relationships. However, a comprehensive phylogenetic study for the genus does not exist. To evaluate the current systematic treatment of the genus, we reconstructed a phylogeny for *Arachis* using nuclear ITS and plastid *trn*T-*trn*F sequences from a total of 48 species representing all nine sections. ITS cloning of the allotetraploid species of section *Arachis* indicated the presence of A and B genome alleles and chimeric sequences. Our study showed species from section *Extranervosae* as the first emerging lineage in the genus, followed by sections *Triseminatae* and *Caulorrhizae,* with two major terminal lineages, which we will refer to as erectoides and arachis. Lineage erectoides comprises members of sections *Erectoides, Heteranthae, Procumbentes, Rhizomatosae,* and *Trierectoides.* Species in the arachis lineage formed two major clades, arachis I (B and D genomes species, and the aneuploids) and arachis II (A genome species). Our results substantiated the sectional treatment of *Caulorrhizae, Extranervosae* and *Triseminatae,* but demonstrated that five sections (*Arachis, Erectoides, Procumbentes,* and *Trierectoides*) are not monophyletic. A detailed study of the genus *Arachis* with denser taxon sampling, additional genomic regions, plus information from morphology and cytogenetics is needed for a comprehensive assessment of its systematics.

Keywords: *Arachis* · peanut · molecular phylogeny · concerted evolution · evolution· systematics

Introduction

The genus *Arachis* (Fabaceae, subfamily Papilionoideae, tribe Dalbergieae) contains approximately 80 species including the economically important cultivated peanut, *A. hypogaea* (Krapovickas and Gregory 1994; Lavin et al. 2001; Valls and Simpson 2005; Wojciechowski et al. 2004). Krapovickas and Gregory (1994) indicated that the most defining morphological features of the genus are the underground structures, including the fruits, rhizomatous structures, root systems, and hypocotyls. Species of *Arachis* are distributed east of the Andes Mountains and south of the Amazon River, with the highest diversity around the eastern border of Bolivia and western Brazil (Krapovickas and Gregory 1994). Gregory et al. (1980) cited central Brazil as the center of origin for *Arachis* and northern Argentina or southern Bolivia as the center of origin of the domesticated peanut. Peanut is among the most important 30 crops that feed the world (Hammer et al. 2003) and is cultivated in more than 100 countries with 37.1 million metric tons produced globally. Other species of *Arachis* have also been cultivated, such as *A. villosulicarpa,* as an indigenous food crop (Galgaro et al. 1997) and *A. glabrata, A. pintoi*, and *A. repens* which are grown as forage crops (Galgaro et al. 1998).

A comprehensive molecular systematic study of the genus is lacking. The latest monograph of *Arachis* was based on morphology, geographic distribution, cytogenetics, and hybridization (Krapovickas and Gregory 1994). It recognized 69 species, dividing them into nine sections: *Arachis, Caulorrhizae, Erectoides, Extranervosae, Heteranthae, Procumbentes, Rhizomatosae, Trierectoides*, and *Triseminatae*. Recently, 11 additional species were recognized in *Arachis* (Valls and Simpson 2005). The boundaries and phylogenetic relationships of the sections are poorly understood. Section (sec.) *Arachis* is the largest, containing 31 species, or 45% of all currently recognized species (Krapovickas and Gregory 1994; Valls and Simpson

2005). It is the most studied of the nine sections because it contains the cultivated peanut and its gene pools (Singh and Simpson 1994). The section is further divided into three groups of species based on their genome type: A, B or D (Smartt et al. 1978; Stalker 1991), with the cultivated *A. hypogaea* and wild *A. monticola* containing AB genomes. The phylogenetic relationships among these species are not fully understood.

A majority of the *Arachis* species are diploid based on $x = 10$ (Krapovickas and Gregory 1994). However, there are five tetraploid species ($2n = 4x = 40$) and four aneuploid species ($x = 9$, $2n = 2x = 18$) (Krapovickas and Gregory 1994; Lavia 1998; Peñaloza and Valls 1997). Two of the tetraploids (*A. hypogaea* and *A. monticola*) are in sec. *Arachis*, and the remaining three are in section *Rhizomatosae* (*A. glabrata, A. pseudovillosa,* and *A. nitida*) (Krapovickas and Gregory 1994; Valls and Simpson 2005). Three of the aneuploids, *A. decora, A. palustris,* and *A. praecox,* belong to section *Arachis* (Lavia 1998; Peñaloza and Valls 1997), but their genomic identity and phylogenetic position has not yet been established. The fourth aneuploid, *A. porphyrocalyx* is placed in sec. *Erectoides* (Peñaloza and Valls 2005).

Krapovickas and Gregory (1994) presented an illustration of intuitive "evolutionary and phylogenetic relationships" among the nine sections of *Arachis* based on information from crossability, morphology and geography. They concluded that *Erectoides, Extranervosae, Heteranthae, Trierectoides* and *Triseminatae* are "older" sections, while *Arachis, Caulorrhizae, Procumbentes,* and *Rhizomatosae* are more "recent" in origin. They indicated that species from section *Erectoides* produce hybrids with members of sections *Arachis, Caulorrhizae, Procumbentes, Rhizomatosae,* and *Trierectoides*. Although most intersectional hybrids are sterile, Krapovickas and Gregory (1994) considered section *Erectoides* to be less isolated than the other sections. Other studies provided cytogenetic evidence for closer genomic relations

between *Erectoides* and the majority of the remaining sections (Gregory and Gregory 1979; Stalker 1981; Valls and Simpson 1994). However, Krapovickas and Gregory (1994) maintained that the use of cytogenetic studies to elucidate the phylogenetic relationships among the sections and species is insufficient because of the genetic barriers that have developed in *Arachis* species from geographic isolation and an autogamous reproductive system.

Relationships among species and some sections of *Arachis* have been evaluated using molecular markers such as restriction fragment length polymorphism (RFLP) and random amplification of polymorphic DNAs (RAPD) (Barkley et al. 2007; Creste et al. 2005; Galgaro et al. 1998; Galgaro et al. 1997; Gimenes et al. 2002b). All of these studies were based on phenetic analyses using the Unweighted Pair Group Methods (UPGMA). Some of these studies supported the Krapovickas and Gregory (1994) classification, while others raised questions concerning the validity of some sections, such as sections *Heteranthae* and *Rhizomatosae.* Hoshino et al. (2006) were the first to represent all nine sections of *Arachis* using microsatellites but, the data were analyzed phenetically with UPGMA. The majority of the species grouped into their respective sections as defined by Krapovickas and Gregory (1994). However, some of sec. *Procumbentes* species grouped with species from section *Erectoides,* while a few grouped with sections *Trierectoides* and *Heteranthae*. Their study also showed that members of sec. *Heteranthae* failed to group together, which is in agreement with the findings of Galgaro et al. (1998).

Two studies used DNA sequence information to examine the relationships among *Arachis* species, but the focus was on section *Arachis* and the likely progenitors of *A. hypogaea* (Jung et al. 2003; Tallury et al. 2005). Jung et al. (2003) used sequences of stearoyl-ACP and oleoyl-PC desaturases from seven species of section *Arachis* and analyzed the data using Maximum Parsimony (MP). Their unrooted tree provided further support for the origin of the A

and B genomes of *A. hypogaea* from *A. duranensis* and *A. ipaensis*, respectively. Tallury et al. (2005) used plastid *trn*T-*trn*F sequences to examine the evolutionary relationships among the genomes in section *Arachis* and the affinities of the aneuploid species to these genomes. Using maximum likelihood (ML), the unrooted tree showed that the B and D genomes are more closely related to each other than to the A genome, and that the aneuploids are closer to the B and D genome species.

We present here a molecular phylogenetic study representing all nine sections and recognized genomes of genus *Arachis* using sequence information from two intergenic spacers (IGS) as well as one intron of the plastid *trn*T-*trn*F region and both nuclear ribosomal internal transcribed spacers (ITS1 & 2). DNA sequence information from these regions has been successfully used to depict the phylogenetic relationships among species within genera of Fabaceae (e.g. Tun and Yamaguchi 2007; Vander Stappen et al. 2002). The data were analyzed phylogenetically using maximum parsimony and Bayesian inference. We also applied TCS network analysis to assess relationships among genomes and alleles within the ITS region due to the presence of diverse sets of haplotypes, including potential chimeras.

Materials and methods

Taxon sampling

We analyzed a total of 48 accessions representing 46 species from all nine sections of *Arachis* recognized by Krapovickas and Gregory (1994). *Stylosanthes humilis and S. fruticosa* were chosen as outgroup species based on their sister group relationship to *Arachis* (Lavin et al. 2001). We generated new ITS and *trn*T-*trn*F sequences for 34 accessions and supplemented the ITS datasets with 14 GenBank sequences. These GenBank sequences did not have

corresponding *trn*T-*trn*F sequences and genomic DNA was not available for them. Consequently, we generated two data sets, one that included the 34 ITS accessions plus four clones we sequenced representing the ITS alleles for the sec. *Arachis* tetraploids, *A. hypogaea* and *A. monticola*. This data set overlaps completely with the *trn*T-*trn*F data set except for the clones. The second data set included the above dataset plus the 14 GenBank sequences for a total of 48 accessions, which we will refer to as the "extended data set." Species examined, sectional affiliation, and sources of material are noted in Table 2.1.

DNA extraction, amplification, cloning, and sequencing

Genomic DNA extraction followed Milla et al. (2005) using fresh leaf material. The ITS region, including ITS1, 5.8S, and ITS2, was amplified using primers ITS4 and ITS5 (White et al. 1990) in a 25 μl reaction mixture containing 1x ThermoPol Buffer (New England Biolabs, Ipswich, MA), 200 μM dNTPs, 20 pmol each primer and 1.5 U *Taq* DNA polymerase (New England Biolabs, Ipswich, MA). The amplifications were carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA) with 2 min initial denaturing at 94° C, 35 cycles of 2 min denaturing at 94° C, 1 min of primer annealing at 52°C, and 1.5 min extension at 72° C, followed by a final extension of 5 min at 72°C.

Amplifications of the *trn*T-*trn*F region were carried out using universal primers (Taberlet et al. 1991) as described in Tallury et al. (2005). Primers rps4-166F and *trn*L-P6/7 (Quandt et al. 2004) were used to generate amplified products when the universal primers failed to amply the *trn*T-*trn*L region. PCR-amplified products for the ITS and *trn*T-*trn*F regions were resolved on 1% TAE-agarose gels containing 0.03 mg of ethidium bromide and were cleaned using QIAquick PCR purification or QIAquick Gel Extraction kits (Qiagen, Valencia, CA). Cycle sequencing reactions were carried out using ABI Big Dye Terminator Ready Reaction kits

(Applied Biosystems Inc., Foster City, CA) following standard protocols and then electrophoresed on an Applied Biosystems 3730 automated sequencer at the Core Sequencing Facility at VBI, Virginia Tech, or at the DNA Analysis Facility of Duke University.

Cloning and sequencing of sec. *Arachis* tetraploid ITS alleles

The amplified ITS region from allotetraploid species *A. hypogaea* and *A. monticola* were ligated into the pGEM-T Easy vector (Promega, Madison, WI) to separate multiple alleles suspected from double peaks in pherograms from direct sequencing of PCR amplifications of genomic DNA. Ligated plasmids were heat shocked into JM109 *E. coli* cells (Promega). Colonies were screened using the blue-white screening method for detecting the insert. Positive clones were grown and DNA was isolated by the plasmid miniprep procedure (Birnboim and Dolye 1979). Inserts were sequenced directly using vector specific primers. When direct sequencing failed, the ITS insert was amplified from the clones using the ITS primers, and the amplified inserts were cleaned and sequenced as detailed above.

Sequence alignment and phylogenetic analyses

Sequences were manually aligned using PhyDE (Müller et al. 2005) following the alignment rules in Kelchner (2000). One identified inversion in the *trn*T-*trn*L spacer was positionally separated in the alignments, and later included as a reverse complement in the phylogenetic analyses, as discussed in Quandt et al. (2003) and Borsch and Quandt (2009). Indels were coded using SeqState (Müller 2005) following the simple indel coding method (Simmons and Ochoterena 2000).

Table 2.1 *Arachis* and outgroup species included in this study.

Genus & Section	Species	Genome	Collector no.	Plant Introduction #	GenBank accession number	
Arachis					ITS	*trn*T-*trn*F
Sec. *Arachis*	*A. batizocoi* Krapov. & W.C. Gregory	B	K 9484	298639	this study	this study
	A. benensis Krapov., W.C. Gregory & C.E. Simpson	A	KGSPSc 35005	475877	this study	this study
	A. correntina (Burkart) Krapov. & W.C. Gregory	A	GKP 9530	262808	this study	this study
	A. cruziana Krapov., W.C. Gregory & C.E. Simpson	B	KSSc 36024	476003	this study	this study
	A. decora Krapov., W.C. Gregory, & Valls		--	--	AY615237	--
	A. diogoi Hoehne	A	GK 10602	--	this study	this study
	A. duranensis Krapov. & W.C. Gregory	A	K 7988	219823	this study	this study
	A. glandulifera Stalker	D	KGSSc 30091	468336	this study	this study
	A. helodes Martius ex Krapov. & Rigoni	A	KG 30029	468144	this study	this study
	A. herzogii Krapov., W.C. Gregory & C.E. Simpson	A	KSSc 36029	476008	this study	this study
	A. hoehnei Krapov. & W.C. Gregory	A	KG 30006	468150	AJ320395	this study
	A. hypogaea L.	AB	--	262090	this study	this study

	A. ipaensis Krapov. & W.C. Gregory	B	KGBSPSc 30076	468322	this study	this study
	A. kempff-mercadoi Krapov., W.C. Gregory & C.E. Simpson	A	KGSSc 30088	468333	this study	this study
	A. kuhlmannii Krapov. & W.C. Gregory	A	VKSSv 8888	--	this study	this study
	A. linearifolia Valls, Krapov. & C.E. Simpson	A	--	--	AY615242	--
	A. magna Krapov., W.C. Gregory & C.E. Simpson	B	KGSSc 30093	468338	AF203555	this study
	A. monticola Krapov. & Rigoni	AB	BaRiK 7264		this study	this study
	A. palustris Krapov., W.C. Gregory, & Valls		VPmSv 13023		this study	this study
	A. praecox Krapov., W.C. Gregory, & Valls		VSGr 6416	476128	this study	this study
	A. schininii Krapov. Valls & C.E. Simpson	A	--	--	AY615248	--
	A. simpsonii Krapov. & W.C. Gregory	A	--	--	AY615247	--
	A. stenosperma Krapov. & W.C. Gregory	A	--	--	AY615252	--
	A. trinitensis Krapov. & W.C. Gregory	A	WiCla 1117	Grif 14278	this study	this study
	A. valida Krapov. & W.C. Gregory	B	VPoBi9157	Grif 7689	this study	this study
	A. williamsii Krapov. & W.C. Gregory	B	WiCla1118	Grif 14229	this study	this study
Sec. *Caulorrhizae*	*A. pintoi* Krapov. & W.C. Gregory	C	18748	604858	this study	this study
	A. repens Handro	C	HLK 467	338277	this study	this study
Sec. *Erectoides*	*A. brevipetiolata* Krapov. & W.C. Gregory	E_2	--	--	AY615251	--

	A. major Krapov. & W.C. Gregory	E_2	HLKHe 559	338294	this study	this study
			GK 10588		this study	this study
	A. paraguariensis Chodat & Hassl.	E_2	GKP 9646	262842	this study	this study
Sec. *Extranervosae*	*A. burchellii* Krapov. & W.C. Gregory	Ex	--	--	AY615262	--
	A. lutescens Krapov. & Rigoni	Ex	--	--	AY615246	--
	A. macedoi Krapov. & W.C. Gregory	Ex	GKP 10127	276203	this study	this study
	A. villosulicarpa Hoehne	Ex	--	--	AY615265	--
Sec. *Heteranthae*	*A. dardani Krapov. & W.C. Gregory*	Am	GK 12943	--	this study	this study
Sec. *Procumbentes*	*A. appressipilia* Krapov. & W.C. Gregory	E_3	GKP 9990	261877	this study	this study
	A. kretschmeri Krapov. & W.C. Gregory	E_3	--	---	AY615220	--
	A. matiensis Krapov., W.C. Gregory & C.E. Simpson	E_3	--	--	AY615249	--
	A. pflugeae C.E. Simpson, Krapov. & Valls	E_3	--	--	AY615233	--
	A. rigonii Krapov. & W.C. Gregory	E_3	GKP 10034	262142	this study	this study
Sec. *Rhizomatosae*	*A. burkartii* Handro	R_1	--	--	AY615245	--
	A. glabrata Benth.	R_2	--	--	AY615250	--
Sec. *Trierectoides*	*A. guaranitica* Chodat & Hassl.	E_1	--	276194	this study	this study
	A. tuberosa Bong. ex Benth.	E_1	--	476142	this study	this study
Sec. Triseminatae	*A. triseminata* Krapov. & W.C. Gregory	T	GK12881	--	this study	this study
			GK 12922	--	this study	this study

Stylosanthes	*S. fruticosa* (Retz.) Alston	--	321413	this study	this study
Stylosanthes	*S. humilis* Kunth	--	275764	this study	this study

The data sets were analyzed with indels coded (IC) as binary characters and without the use of indels (IN). Aligned datasets for the nuclear ITS (excluding the 26S exon) and plastid *trn*T-*trn*F were analyzed separately and in combination.

Since in *Arachis* the plastid genome follows a maternal inheritance (Tallury et al. 2005), only the maternal ITS region as identified by the TCS analysis was used for the allotetraploid species in the combined analysis. Phylogenetic analyses of the three data sets were conducted using maximum parsimony in PAUP* version 4.0b10 (Swofford 2003) and Bayesian inference (BI) in MrBayes (Huelsenbeck and Ronquist 2001). Maximum Parsimony (MP) analyses were performed as heuristic tree searches using tree bisection-reconnection (TBR) branch-swapping with 100 random addition sequence replicates, and incorporating the parsimony ratchet algorithm (Nixon 1999) via PRAP2 (Müller 2007). A strict consensus tree was generated for each data set. Support for the clades was obtained by performing bootstrap (BS; Felsenstein 1985) searches with 100 replicates and 10 random sequence replicates. Bayesian analyses were performed using the GTR+Γ+I models, with 2,000,000 generations and 10 runs with 4 chains. Trees were compiled using TreeGraph2 (Müller and Müller 2004; Stöver and Müller 2009).

A partition homogeneity test (PHT; Farris et al. 1995) was performed in PAUP* (Swofford 2003) prior to the combined analyses to determine if the ITS and *trn*T-*trn*F regions were congruent in their mode of evolution. The PHT was conducted using 100 replicate partitions. For each replicate partition, no more than 500 trees were saved with a score greater than 1. The resulting P-value (0.01) indicate the two regions are incongruent, which implied that the ITS and *trnT*-trn*F* regions should not be combined. However, PHT has been found to be sensitive to noise and different mutation rates between genomic regions, and might not be an accurate measure of congruence between regions (Baker and Lutzoni 2002; Darlu and Lecointre

2002; Dolphin et al. 2000). To assess the potential sources of conflict, the matrices for the two genomic regions were analyzed separately and in combination. Comparison of trees derived from the partitioned analyses showed that support for conflicting nodes was low, implying soft incongruence (Johnson and Soltis 1998).

Haplotype networks were constructed using TCS (Clement et al. 2000; Clement et al. 2002). Because only singleton gaps were observed in the data, the probability of parsimony was set to 95% cut off. The TCS analysis allowed for the identification of chimeric ITS sequences that were subsequently excluded from phylogenetic analyses.

Results

ITS alleles and sequence statistics

Amplification and sequencing of the ITS region generated a single homogenous sequence in each case with the exception of the two allotetraploid species *A. hypogaea* and *A. monticola.* In these allotetraploids, although the initial reading of the chromatograms depicted a prominent signal for the putative A genome allele, re-examination revealed the presence of secondary peaks and what appeared as possible background noise (Figure 2.1). Consequently, the genomic ITS PCR products of the tetraploids were cloned and sequenced. A total of 14 clones each from *A. hypogaea* and *A. monticola* were obtained. For *A. hypogaea*, the majority of the clones corresponded to the ITS sequence of the A genome species (10 clones; 71.4%) while two clones matched the B genome species (14.3%). The remaining two clones were chimeric as noted below. In case of *A. monticola*, six clones (42.9%) represented the A genome, whereas two clones (14.3%) represented the B genome. The remaining six clones were chimeric as noted below. The majority of the clones with the A genome clustered with the *A. duranensis*

haplotype, while those with the B genome grouped with the *A. ipaensis* haplotype in the TCS analysis (Figure 2.2). There were a few clones in which the haplotype differed from either *A. duranensis* or *A. ipaensis* due to mutations in a few positions (Figure 2.2). When the alignment of the A and B genome clones was examined, the positions where mutational differences were observed corresponded exactly to the position in the alignment of the genomic sequences where double peaks were detected. The two alleles differed by eight substitutions and a single-base indel. The lower frequency of the B allele in the allotetraploids may have caused the lower signal (smaller peaks) in the chromatograms, whereas the one-base gap resulted in a chromatogram shift that appeared as background noise (Figure 2.1).

Alleles with chimeric sequences (total of 7 clones, 24%) were also observed in both allotetraploids. These included three types of chimera that differ in the genomic identity of ITS1 and ITS2 regions: 1) ITS1 represents the A allele, while ITS2 represents the B allele (2 clones, 14.3%), 2) ITS1 represents the B allele, while ITS2 represents the A allele (1 clone, 7.1%), and 3) one of the ITS regions is a hybrid of the A and B genomes, whereas the other represents one of the two genomes (5 clones; 35.7%). The positions of the chimeric clones in relation to the A and B genome haplotypes are illustrated in the TCS network (Figure 2.2).

ITS based phylogeny

The complete ITS region sequences for the genus varied in length from 599 in *A. brevipetiolata* to 657 bp in *A. hypogaea*. The sequences were trimmed at the 26S rDNA subunit, 63 bp, as these sequences were incomplete for this exon. Twelve indels of 1-9 bp were required for the alignment of all the species including the outgroup taxa, resulting in a total alignment of 614 characters. The ITS dataset consisted of 38 taxa, 36 of which were included in the ingroup.

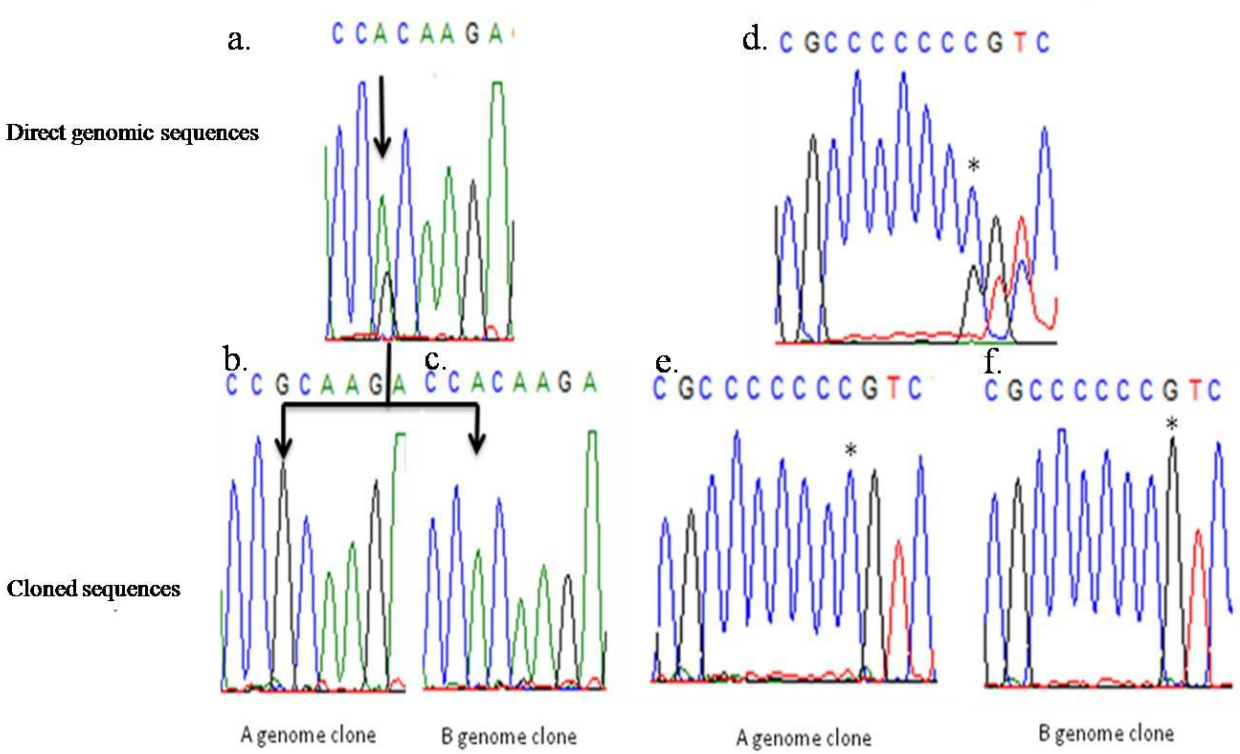

Figure 2.1 Examples of chromatograms of sec. *Arachis* allotetraploids ITS sequences showing double peaks and a singleton base-pair insertion. a. An arrow marks a double peak in a genomic DNA sequence. b-c. Arrows mark corresponding positions from ITS sequences of cloned PCR product of *A. hypogaea* and *A. monticola*. d. Direct genomic sequencing chromatogram showing a double peak, marked by asterisk, indicative of a shift in the sequence due to a gap. e-f. Nucleotide present at that position in cloned sequences from the allotetraploids marked with an asterisk.

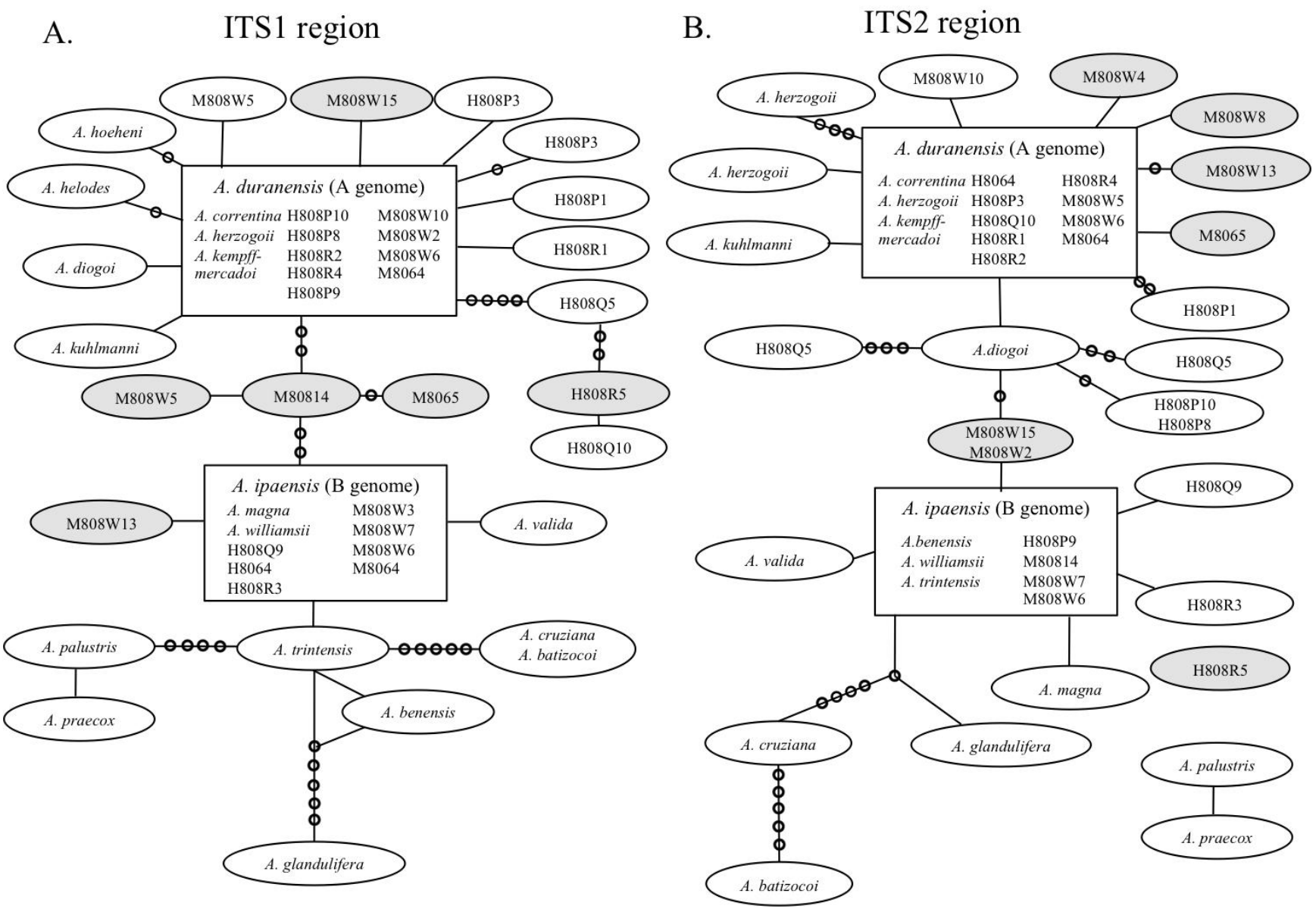
A.
ITS1 region
M808W5
M808W15
H808P3
A. hoeheni
H808P3
A. helodes
A. duranensis (A genome)
A. correntina H808P10 M808W10
A. herzogoii H808P8 M808W2
A. kempff-mercadoi H808R2 M808W6
H808R4 M8064
H808P9
H808P1
A. diogoi
H808R1
H808Q5
A. kuhlmanni
M808W5
M80814
M8065
H808R5
H808Q10
A. ipaensis (B genome)
A. magna M808W3
A. williamsii M808W7
H808Q9 M808W6
H8064 M8064
H808R3
M808W13
A. valida
A. palustris
A. trintensis
A. cruziana
A. batizocoi
A. praecox
A. benensis
A. glandulifera
B.
ITS2 region
A. herzogoii
M808W10
M808W4
M808W8
A. duranensis (A genome)
A. correntina H8064 H808R4
A. herzogoii H808P3 M808W5
A. kempff-mercadoi H808Q10 M808W6
H808R1 M8064
H808R2
A. herzogoii
M808W13
A. kuhlmanni
M8065
H808P1
H808Q5
A.diogoi
H808Q5
H808P10
H808P8
M808W15
M808W2
H808Q9
A. ipaensis (B genome)
A.benensis H808P9
A. williamsii M80814
A. trintensis M808W7
M808W6
A. valida
H808R3
H808R5
A. magna
A. cruziana
A. glandulifera
A. palustris
A. praecox
A. batizocoi

Figure 2.2 Haplotype networks for sec. *Arachis* diploid species and clones for the allotetraploids *A. hypogaea* (H) and *A. monticola* (M): **a.** ITS1 and **b.** ITS2 regions. Clones that contained chimeric haplotypes are shaded in gray.

For the MP analyses of the non-indel-coded dataset, of the 614total characters analyzed 153 (25%) were variable and 97 (63.4%) were parsimony informative. Based on this dataset, six trees were generated with a length of 196 steps. The consistency index (CI) was 0.913 and retention index (RI) was 0.938. For the indel-coded dataset, 174 (27.4%) of the total 634 characters were variable, and of those, 108 (62.1%) were parsimony informative. Analysis of the indel-coded ITS dataset generated seven trees with a length of 221 steps. The CI and RI for this dataset were 0.905 and 0.932, respectively. The Bayesian analyses with and without indels coded generated 50% majority trees that were congruent in topology and identical to the MP consensus trees, differing only in degrees of support for some of the nodes (Figure 2.3). Since topology was the same for the MP and BI trees, the BI tree is presented here with bootstrap (BS) and posterior probability (PP) support values noted on the branches.

The monophyly of the genus received full BS and PP supported with (IC) and without (IN) indels coded as characters (Figure 2.3). The first deriving lineage was sec. *Extranervosae* represented by *A. macedoi*, excluded from the rest of the genus with low BS support (62% IN, 54% IC). When analyzed using BI, *A. macedoi* was excluded from the remaining *Arachis* species with significant posterior probability (PP) without indels coded as characters (0.95), but the exclusion of this species lost significant support when indels were included as characters (0.89). Accessions of the monotypic sec. *Triseminatae* were the next to diverge and received full support in all analyses. Section *Triseminatae* was excluded from the remaining *Arachis* species by strong to full support in all analyses (99% BS-IN, 100% BS-IC, 1.00 PP-IN, 1.00 PP-IC). The next diverging lineage comprised of species of sec. *Caulorrhizae* (*A. pintoi* and *A. repens*), receiving strong support (95% BS-IN, 99% BS-IC, 1.00 PP-IN, 1.00 PP-IC). Section

Caulorrhizae was excluded from the remaining two lineages with moderate BS support (76% IN, 68% IC) in the MP analyses and full PP support in the BI analyses.

The other terminal lineage consisted of sec. *Arachis* species divided into two clades (Figure 2.3), which we will refer to as arachis I and arachis II. The monophyly of this lineage received moderate support (68% BS-IN, 71% BS-IC), but significant PP support (0.97 IN, 0.96 IC). Group arachis I comprised of B genome, D genome, and aneuploid species, and received strong BS support (98% BS-IN, 98% BS-IC) and full PP support. Two subclades were resolved within group arachis I. The first consisted of the aneuploid species *A. praecox* and *A. palustris*, receiving full support in all analyses. The second subclade included B and D genome species, receiving 86% BS-IN and 87% BS-IC and strong PP (0.99 IN, 0.99 IC). Species within this subclade appeared in two polytomies, one included *A. batizocoi* + *A. cruziana* (100% BS-IN, 100% BS-IC, 1.00 PP-IN, 1.00 PP-IC), *A. benensis, A. glandulifera* (D genome), *A. trinitensis* (B genome), and the other (65% BS-IN, 63% BS-IC) comprised of *A. ipaensis, A. magna, A. williamsii, A. valida,* and the tetraploid B genome clones. Group arachis II (66% BS-IN, 67% BS-IC, 0.65 PP-IN, 0.86 PP-IC) comprised of a polytomy of *A. major* 10588 (sec. *Erectoides*), *A. hoehnei* and *A. diogoi* plus a subclade of yet another polytomy (61% BS-IC, 0.92 PP-IC) of Section *Arachis* A genome species *A. correntina, A. duranensis, A. herzogii, A. kempff-mercadoi, A. kuhlmannii,* and clones representing the A genome from the tetraploid species. When indels were excluded from analyses, a total polytomy was evident.

In the extended ITS dataset, a total of 619 characters were analyzed using MP and BI, of these 171 (27.6%) were variable and 117 (68.4%) parsimony informative. Fifteen trees most parsimonious were obtained with a length of 238 steps, CI of 0.861, and RI of 0.929.

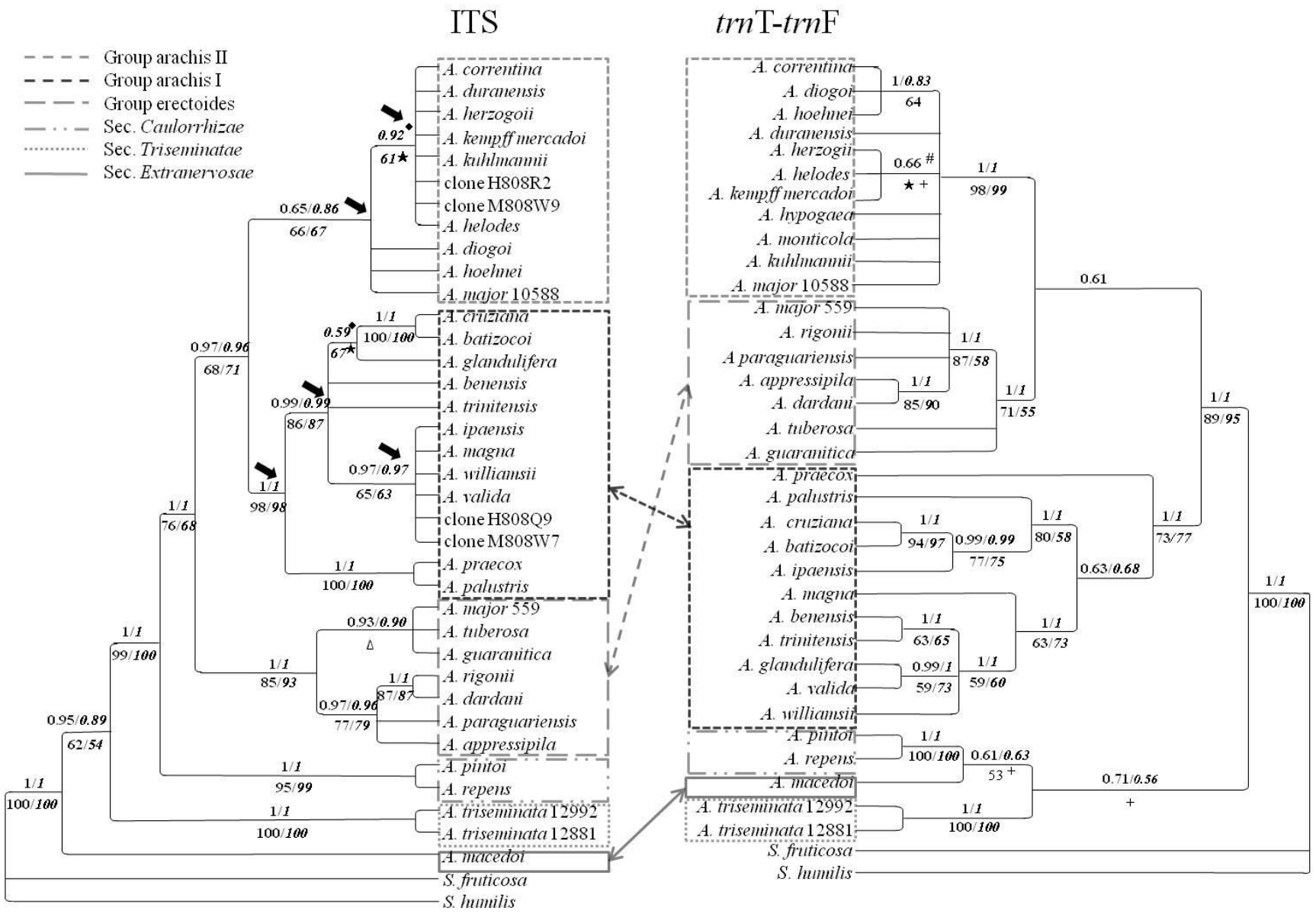
ITS
trnT-trnF
Group arachis II
Group arachis I
Group erectoides
Sec. Caulorrhizae
Sec. Triseminatae
Sec. Extranervosae
A. correntina
A. duranensis
A. herzogoii
A. kempff mercadoi
A. kuhlmannii
clone H808R2
clone M808W9
A. helodes
A. diogoi
A. hoehnei
A. major 10588
A. cruziana
A. batizocoi
A. glandulifera
A. benensis
A. trinitensis
A. ipaensis
A. magna
A. williamsii
A. valida
clone H808Q9
clone M808W7
A. praecox
A. palustris
A. major 559
A. tuberosa
A. guaranitica
A. rigonii
A. dardani
A. paraguariensis
A. appressipila
A. pintoi
A. repens
A. triseminata 12992
A. triseminata 12881
A. macedoi
S. fruticosa
S. humilis
A. correntina
A. diogoi
A. hoehnei
A. duranensis
A. herzogii
A. helodes
A. kempff mercadoi
A. hypogaea
A. monticola
A. kuhlmannii
A. major 10588
A. major 559
A. rigonii
A paraguariensis
A. appressipila
A. dardani
A. tuberosa
A. guaranitica
A. praecox
A. palustris
A. cruziana
A. batizocoi
A. ipaensis
A. magna
A. benensis
A. trinitensis
A. glandulifera
A. valida
A. williamsii
A. pintoi
A. repens
A. macedoi
A. triseminata 12992
A. triseminata 12881
S. fruticosa
S. humilis

Figure 2.3 Bayesian inference 50% phylogeny majority rule consensus trees for *Arachis* rooted with *Stylosanthes humilis* and *S. fruticosa* and used GTR+Γ+I model based on sequences from nuclear ribosomal ITS region (left) and *trn*T-*trn*F region (right). Arrows indicate branches that collapsed when the IUPAC characters were included for the allotetraploid ITS sequences in the dataset. Δ = branches were not recovered in MP analyses. + = branches not recovered in MP analyses with indel coding ★= branches not recovered in MP analyses without indel coding. ◆ = branches not recovered in the BI analysis when indel characters were included. Posterior probabilities are above the branches and bootstrap support values are below. Supports with indels coded are on the left while those obtained without indel coding are on the right.

When indels were included, the total of 652 characters contained 204 (31.3%) that were variable, of which 133 (65.2%) were parsimony informative. The MP analyses generated 58 trees with a length of 277 steps. The CI and RI were 0.859 and 0.925, respectively. The topology of the consensus trees was the same with and without indel coding, differing in resolution within group arachis I and arachis II (Figure 2.4).

As in the trees based on the non-extended ITS dataset, the monophyly of the genus was fully supported (Figure 2.4). The first deriving lineage consisted of species from sec. *Extranervosae* and was fully supported in all analyses. This lineage formed two clades, one clade included the species *A. macedoi* and *A. lutescens* (94% BS-IN, 98% BS-IC, 1.00 PP-IN, 1.00 PP-IC) and the other *A. burchellii* and *A. villosulicarpa* (92% BS-IN, 99% BS-IC, 1.00 PP-IN, 1.00 PP-IC). The exclusion of sec. *Extranervosae* from the remaining species of *Arachis* received moderate BS support (79% IN, 68% IC) and significant PP support (1.00 IN, 0.99 IC). Accessions of the monotypic sec. *Triseminatae* were the next to diverge and received full support in all analyses (Figure 2.4). The two basal lineages were excluded from the rest of the genus with very strong BS support (99% IN, 99% IC) and full PP support. The monophyly of sec. *Caulorrhizae* was also strongly supported (93% IN, 99% IC, full PP support; however, their exclusion from the remaining *Arachis* lineages receive <50% BS support and lacked significant PP support (0.74 IN, 0.75 IC). The diploid species of sec. *Rhizomatosae*, *A. burkartii* diverged next. The exclusion of *A. burkartii* from remaining *Arachis* was less than 50% BS with or without indels coded as characters and received slightly less than significant PP support (0.94); however, the exclusion of this species did receive significant support (0.96) when indels were not coded as characters in the BI analysis.

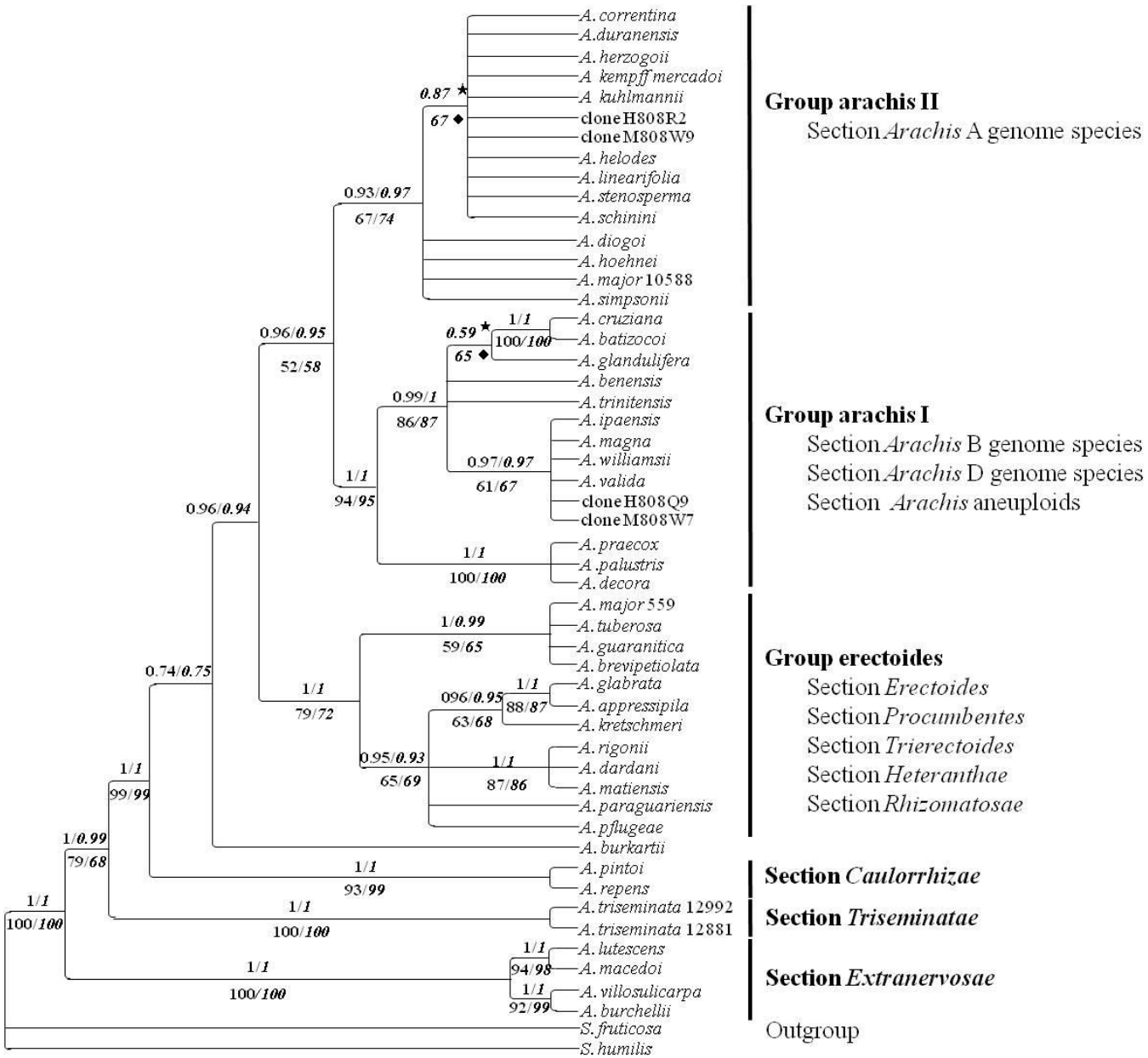
A. correntina
A.duranensis
A. herzogoii
A kempff mercadoi
A kuhlmannii
clone H808R2
clone M808W9
A. helodes
A. linearifolia
A. stenosperma
A. schinini
A. diogoi
A. hoehnei
A. major 10588
A. simpsonii
A. cruziana
A. batizocoi
A. glandulifera
A. benensis
A. trinitensis
A. ipaensis
A. magna
A. williamsii
A. valida
clone H808Q9
clone M808W7
A. praecox
A .palustris
A. decora
A. major 559
A. tuberosa
A. guaranitica
A. brevipetiolata
A. glabrata
A. appressipila
A. kretschmeri
A. rigonii
A. dardani
A. matiensis
A. paraguariensis
A. pflugeae
A. burkartii
A. pintoi
A. repens
A. triseminata 12992
A. triseminata 12881
A. lutescens
A. macedoi
A. villosulicarpa
A. burchellii
S. fruticosa
S. humilis
0.87 ★
67 ◆
0.93/0.97
67/74
0.59 ★
65 ◆
1/1
100/100
0.96/0.95
52/58
0.99/1
86/87
0.97/0.97
61/67
1/1
94/95
0.96/0.94
1/1
100/100
1/0.99
59/65
0.74/0.75
1/1
79/72
096/0.95
63/68
1/1
88/87
0.95/0.93
65/69
1/1
87/86
1/1
99/99
1/0.99
79/68
1/1
93/99
1/1
100/100
1/1
100/100
1/1
94/98
1/1
92/99
Group arachis II
Section Arachis A genome species
Group arachis I
Section Arachis B genome species
Section Arachis D genome species
Section Arachis aneuploids
Group erectoides
Section Erectoides
Section Procumbentes
Section Trierectoides
Section Heteranthae
Section Rhizomatosae
Section Caulorrhizae
Section Triseminatae
Section Extranervosae
Outgroup

Figure 2.4 *Arachis* phylogeny generated using Bayesian inference based on ITS expanded dataset, indels coded as characters, and GTR+Γ+I model. Phylogenies generated using MP were nearly identical to the Bayesian tree in topology. + = branches not recovered in MP analyses with indel coding ★= branches not recovered in MP analyses without indel coding. ◆ = branches not recovered in the BI analysis when indel characters were included. Posterior probabilities are above the branches and bootstrap support values are below. Supports with indels coded are on the left while those obtained without indel coding are on the right.

The sections represented in each of the terminal lineages had similar composition when compared to trees based on the nonextended ITS (Figure 2.4), with the exception of the inclusion of the sec. *Rhizomatosae* tetraploid species *A. glabrata* with group erectoides. Within group erectoides (79% BS-IN, 72% BS-IC, 1.00 PP-IN, 1.00 PP-IC), two clades were once again resolved. The first clade included sec. *Trierectoides* and two species of sec. *Erectoides*, *A. major* 559 and *A. brevipetiolata*, in a polytomy that received low BS support (59% IN, 65% IC) but significant to full PP support (1.00 IN, 0.99 IC). The second clade was more resolved with *A. paraguariensis* (sec. *Erectoides*) and *A. pflugeae* (sec. *Procumbentes*) sister to two subclades. In the first subclade (63% BS-IN, 68% IC, 0.96 PPP-IN, 0.95 PP-IC), *A. kretschmeri* appeared sister to *A. glabrata* (sec. *Rhizomatosae*) plus *A. appressipilia* (sec. Procumbentes; 88% BS-IN, 87% BS-IC, 1.00 PP-IN, 1.00 PP-IC). The second subclade comprised of *A. rigonii, A. dardani,* and *A. matiensis* in a polytomy that received good BS support (87% IN, 86% IC) and full PP support (1.00 IN, 1.00 IC).

In the other terminal lineage in the tree based on the extended ITS, sec. *Arachis* species were resolved into groups, arachis I and arachis II (Figure 2.4). The whole lineage received low BS support (52% IN, 58% IC), but significant PP support (0.96 IN, 0.95 IC). As in the nonextended dataset, group arachis I received strong BS (94% BS-IN, 95% BS-IC) and full PP support. Similar to the nonextended dataset, the aneuploids subclade received full support in all analyses, and included the aneuploid species *A. decora*, which was not represented in the nonextended dataset. The structure of the other subclade containing the B and D genome species was the same as in the tree based on the nonextednded dataset (86% BS-IN, 87% BS-IC, 0.99 PP-IN, 1.00 PP-IC). The polytomy that contained *A. ipaensis, A. magna, A. williamsii, A. valida*, and the tetraploid clones had low BS support (61% IN, 67% IC) and significant PP support (0.97

IN, 0.97 IC). While *A. batizocoi* and *A. cruziana* had full support in all analyses (100% BS-IN, 100% BS-IC, 1.00 PP-IN, 1.00 PP-IC), the sister relationship of *A. glandulifera* to these two B genome species was identified only in analyses using indels as characters. However, it lacked significant PP support (0.59 PP-IC) and had low BS support (65% IC). Support for analyses in which indels were not used as characters, had less than 50% support values for BS and PP. The other clade, group arachis II (within the sec. *Arachis* lineage) contained the A genome species and clone sequences as previously identified. Group arachis II included *A. linearifolia, A. stenosperma, A. schininii,* and *A. simpsonii* in a polytomy (67% BS-IN, 74% BS-IC, 0.93 PP-IN, 0.97 PP-IC) with the other A genome species as in the phylogenies based on the initial ITS dataset.

trnT-trnF based phylogeny

The sequences of the *trn*T-*trn*F region varied from 1789 to 1960 bp in length. Seventeen indels of 1-22 bp were coded in the alignment, resulting in a matrix of 2052 characters. From the 5' *trn*L exon, 117 characters were excluded from the analyses due to the presence of a large amount of missing data. Consequently, 1935 characters were analyzed, of which 202 (10.4%) were variable and 114 (56.4%) parsimony informative. The *trn*T-*trn*F dataset generated 31 most parsimonious trees with a length of 241 steps with CI and RI values of 0.892 and 0.912, respectively. When indels were coded as characters for the reduced data set, the total number of characters increased to 1995, with 261 (13.1%) characters being variable and 152 (58.2%) parsimony informative. From the indel-coded dataset, 391 most parsimonious trees were produced of 336 steps with CI and RI values of 0.815 and 0.854, respectively. The phylogenetic trees obtained from the MP analyses without indels coded and BI analyses with and without

indel coded produced congruent trees, differing only in degrees of support for the nodes (Figure 2.3). The phylogeny produced in MP analyses with indel coding was more resolved at the base, but had low BS support and lacked PP support. The phylogenetic structure of the terminal lineages was consistent with that resulting from the MP without indels coded and all BI analyses.

The monophyly of the genus received full support in all analyses. Based on the plastid sequence data, two major lineages were recovered in the Bayesian analyses (Figure 2.3), with and without indel coding, and the MP analysis without indel coding. The phylogeny generated using MP analysis with indels coded as characters did not recover the first lineage and had a backbone topology that was more like the ITS based phylogenies, but lacked support (data not shown). Thus, the *trn*T-*trn*F based phylogeny recovered from the BI analyses and MP analysis with indels as characters is shown and discussed here (Figure 2.3). The first lineage comprised of three sections corresponding to the first three diverging lineages in the ITS based phylogenies, *Caulorrhizae*, *Extranervosae*, and *Triseminatae*. However, this lineage did not receive BS support above 50% nor did it receive significant PP support (0.71 IN, 0.56 IC). The three sections resolved in a grade corresponding to accessions of the monotypic sec. *Triseminatae* (100% BS-IN, 100% BS-IC, 1.00 PP-IN, 1.00 PP-IC), followed by *A. macedoi* (sec. *Extranervosae*), and then sec. *Caulorrhizae* (100% BS-IN, 100% BS-IC, 1.00 PP-IN, 1.00-PPIC). The exclusion of sec. *Triseminatae* from remaining species within this lineage lacked significant support (53% BS-IN, <50% BS-IC0.61 PP-IN, 0.63 PP-IC).

The second lineage comprised of species in groups arachis I, arachis II, and erectoides, and received strong BS (89% IN, 95 IC) and full PP support (1.00 IN, 1.00 IC; Figure 2.3). Group arachis II resolved sister to groups arachis I plus erectoides; however, the sister relationship between the latter two clades lacked significant PP support and received <50% BS

regardless of indel coding. Group arachis II received moderate BS support (73% IN, 77%IC) and full PP support (1.00 IN, 1.00 IC). The aneuploid species *A. praecox* was sister to the remaining species of this group, though its exclusion received less than 50% BS support (IN and IC) and lacked PP support (0.63 IN, 0.68 IC). Following *A. praecox*, the species were divided into two subclades. The first subclade (80% BS-IN, 58% BS-IC, 1.00 PP-IN, 1.00 PP-IC) consisted of a grade of the aneuploid *A. palustris*, followed by *A. ipaensis* (77% BS-IN, 75% BS-IC, 0.99 PP-IN, 0.99 PP-IC), and *A. cruziana* plus *A. batizocoi* (94% BS-IN, 97% BS-IC, 1.00 PP-IN, 1.00 PP-IC). In the second group, which represents the arachis I subclade (63% BS-IN, 73% BS-IC, 1.00 PP-IN, 1.00 PP-IC), *A. magna* came sister to a polytomy (59% BS-IN, 60% BS-IC, 1.00 PP-IN, 1.00 PP-IC) that was comprised of *A. benensis* plus *A. trinitensis* (63% BS-IN, 65% BS-IC, 1.00 PP-IN, 1.00 PP-IC), *A. glandulifera* plus *A. valida* (59% BS-IN, 73% BS-IC, 0.99 PP-IN, 1.00 PP-IC), and *A. williamsii.* Group erectoides was the second clade resolved here (71%BS-IN, 55% BS-IC, 1.00 PP-IN, 1.00 PP-IC) and reflects the same sections and species composition as the ITS based phylogeny. The two species of sec. *Trierectoides* formed a polytomy and were sister to another polytomy comprising the remaining species (87% BS-IN, 58% BS-IC, 1.00 PP-IN, 1.00 PP-IC). *Arachis appressipilia* and *A. dardani* resolved together with good BS support (85% IN, 90% IC) and full PP support. Group arachis II was comprised of a polytomy of *A. major* (10588), *A. kuhlmannii*, *A. monticola, A. hypogaea, A. duranensis* (98% IN, 99% IC, full PP support) plus two subclades of *A. correntina*/*A. diogoi/A. hoehnei* (64% BS-IN, <50% BS-IC, 1.00 PP-IN, 1.00 PP-IC), and *A. herzogii/A. helodes/A. kempff-mercadoi* (0.66 PP-IN) were resolved in polytomies.

Phylogenies based on combined ITS and *trn*T-*trn*F data

The MP analyses of the combined dataset included 2547 total characters, of which 355 (13.9%) were variable and included 211 (59.4%) parsimony informative characters. This analysis resulted in 167 most parsimonious trees of 446 steps. The CI was 0.883 and RI was 0.909. When indels were coded, the dataset expanded to 2628 characters. The combined indel-coded dataset had 260 (60%) parsimony informative characters from the 435 (16.6%) variable characters. The MP analyses of the indel-coded dataset generated 540 most parsimonious trees of 568 steps. The CI and RI were 0.835 and 0.872, respectively. Strict consensus trees from both MP and BI analyses were identical in topology (Figure 2.5), but differed in PP and BS support values.

The monophyly of the genus based on combined ITS and *trn*T-*trn*F sequence data was fully supported in all analyses (Figure 2.5). At the base of the *Arachis* phylogeny, a grade emerged representing *Arachis macedoi* (sec. *Extranervosae*), followed by sec. *Triseminatae*, then sec. *Caulorrhizae*, then a clade of group erectoides, and groups arachis I and arachis II. The exclusion of *A. macedoi* from the remaining *Arachis* species received moderate BS support (73% IN, 79% IC) and significant PP support (0.97 IN, 0.98 IC), while the exclusion of *A. macedoi* and sec. *Triseminatae* received strong BS support (98% IN, 98% IC) and full PP support.

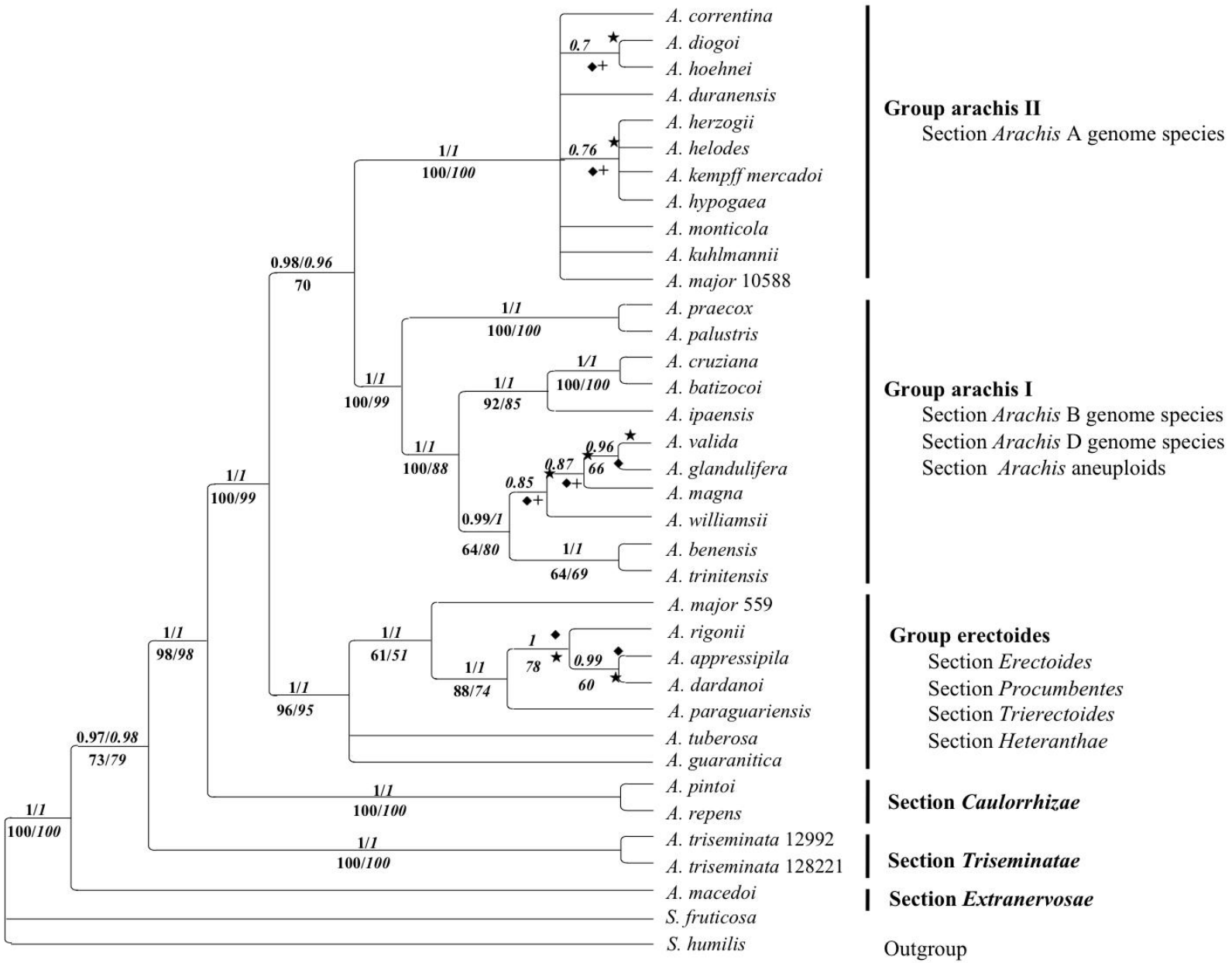
A. correntina
A. diogoi
A. hoehnei
A. duranensis
A. herzogii
A. helodes
A. kempff mercadoi
A. hypogaea
A. monticola
A. kuhlmannii
A. major 10588
A. praecox
A. palustris
A. cruziana
A. batizocoi
A. ipaensis
A. valida
A. glandulifera
A. magna
A. williamsii
A. benensis
A. trinitensis
A. major 559
A. rigonii
A. appressipila
A. dardanoi
A. paraguariensis
A. tuberosa
A. guaranitica
A. pintoi
A. repens
A. triseminata 12992
A. triseminata 128221
A. macedoi
S. fruticosa
S. humilis
0.7
1/1
100/100
0.76
0.98/0.96
70
1/1
100/99
1/1
100/100
1/1
92/85
1/1
100/88
0.96
66
0.87
0.85
0.99/1
64/80
1/1
64/69
1/1
100/99
1/1
98/98
1/1
61/51
1
78
0.99
60
1/1
88/74
1/1
96/95
0.97/0.98
73/79
1/1
100/100
1/1
100/100
1/1
100/100
Group arachis II
Section Arachis A genome species
Group arachis I
Section Arachis B genome species
Section Arachis D genome species
Section Arachis aneuploids
Group erectoides
Section Erectoides
Section Procumbentes
Section Trierectoides
Section Heteranthae
Section Caulorrhizae
Section Triseminatae
Section Extranervosae
Outgroup

Figure 2.5 *Arachis* phylogeny based on combined ITS and *trn*T-*trn*F datasets analyzed with BI, indels included as characters. Branches not resolved in phylogenies generated using MP without indels coded are noted with a star and those not resolved in BI without indel coding are noted with a diamond. Branches that were not resolved in MP with indels coded as characters are indicated by a plus sign. Posterior probabilities are above branches, without indels coded as characters on the left and values with indels used as characters noted on the right. Bootstrap values are below each branch and have BS values on the left without indels as characters while values with indel coding are on the right.

Exclusion of sec. *Caulorrhizae* from the three latter groups received strong to full support (100% BS-IN, 99% BS-IC, 1.00 PP-IN, 1.00 PP-IC). Species topology within the strongly supported group erectoides (96% BS-IN, 95% BS-IC, 1.00 PP-IN, 1.00 PP-IC) was similar to the *trn*T-*trn*F based tree. Group arachis I comprised of two subclades similar in topology to the ITS tree. The exceptions were the placement of *A. ipaensis* and *A. glandulifera* within this subclade (Figure 2.5). Species composition and topology for group arachis II was consistent with phylogenies based on the partitioned ITS and *trn*T-*trn*F dataset and received full support in all analyses.

Discussion

Molecular Evolution

Considering the average length of 629 bp for the ITS region, the number of variable characters was 153, or 24.3%. In contrast, the *trn*T-*trn*F region was 1914 bp in average length with 202 variable characters or 10.4%. Therefore, it appears that the ITS region has a higher rate of substitution than the *trn*T-*trn*F in *Arachis*. In other words, for sequencing three-fold the length of the ITS region, we obtained only 24.2% more variable sites from the *trn*T-*trn*F. Of these variable sites, the number of potentially parsimony informative sites from the two regions were similar, 97 PI for ITS vs. 114 for the *trn*T-*trn*F. The CI and RI values calculated on the trees based on the ITS region were slightly higher than those based on the *trn*T-*trn*F, implying higher proportion of homoplasy in the latter ($p < 0.05$). When the robustness of the phylogenetic trees obtained from these two genomic regions is compared, it appears that the two regions produced comparable number of resolved nodes and overall PP and BS support. Therefore, despite the low proportion of variable and PI sites in the *trn*T-*trn*F compared with the ITS and

the slightly higher homoplasy, the two regions provided comparable phylogenetic information in *Arachis* phylogenetic reconstruction.

Generally, the ITS region is thought to evolve towards one of the parental progenitors in hybrids and allopolyploids because it undergoes rapid homogenization (Álverez and Wendel, 2003; Soltis et al., 2008). Considering the signal in the chromatogram obtained from the direct genomic sequencing of the allotetraploids species *A. hypogaea* and *A. monticola*, the A genome ITS allele appeared to be the more dominant in positions where double peaks representing the two alleles were found. This may be explained by the presence of the A allele clones at much higher frequency than the B allele clones (71.4% vs. 14.3% for *A. hypogaea* and 42.9% vs. 14.3% for *A. monticola*). This implies that the ongoing process of concerted evolution is favoring the A allele over the B allele. Although the occurrence of multiple alleles has been frequently documented (e.g. Kovarik et al., 2004; Soltis et al., 2008; Vander Stappen et al., 1998), their detection can sometimes go unnoticed since the region contains few polymorphic sites indicative of each genome (Soltis et al., 2008), or low signal exists for one of them as shown in this study. This study has also shown the presence of chimeric alleles in six of the 14 (42.8%) *A. monticola* clones, and 2 of the 14 *A. hypogaea* clones (14%), implying the occurrence of infra-allelic recombination events in the ITS of both tetraploid genomes. The presence of chimeric alleles was confirmed in our TCS analysis of the ITS1 and ITS2 alleles from diploid species in sec. *Arachis* and the cloned alleles from the tetraploid species (Figure 2.2). These chimeric alleles were closely linked with their respective ITS regions of their parental alleles as demonstrated by the TCS haplotype network (Figure 2.2). This evolutionary dynamic in the *Arachis* ITS region as illustrated by the chimeric ITS may represent a fluid state

that will subsequently undergo fixation by concerted evolution towards one of the ancestral genotypes.

Without cloning, nucleotide sites showing multiple peaks in the direct genomic sequences of taxa with multiallelic ITS regions are usually assigned IUPAC ambiguity codes. Soltis et al. (2008) have deomnstrated that phylogenetic reconstruction will be negatively impacted when IUPAC ambiguity codes are used as molecular characters. This point received further support from this study. The inclusion of the nine IUPAC coded characters for the allotetraploid species in a dataset that has a total of 153 variable characters and 97 PI characters resulted in considerable collapse of nodes in the clades containing the tetraploids (arachis I and arachis II clades; Figure 2.3). However, when cloned sequences representing the A and B alleles were used alone to represent the tetraploid species in the MP analyses, resolution improved significantly and the clones grouped with the sequences of the respective putative diploid progenitors (Figure 2.3). The collapse of nodes illustrates the impact of ambiguous sequences due to multiple copies not previously recognized in the genome and stresses the point of how carefully ITS data should be treated in general. Once ambiguous sites are detected then cloning of this region is highly recommended.

Phylogeny of the genus *Arachis*

Analyses of the partitioned ITS and *trn*T-*trn*F regions produced alternative topologies (Figures 2.4, 2.5). The most notable was the resolution at the base and the placement of group erectoides. The three basal lineages in the ITS based phylogenies (Figure 2.4) appeared in a single clade in most analyses of the *trn*T-*trnF* data (Fig 2.5). However, the monophyly of this lineage was <50% BS and lacked significant PP support. The difference in topology at the base

of the phylogeny can thus be regarded as soft incongruence. The structure of the terminal lineages and clades in the phylogeny varied depending on the dataset used (Figures 2.4, 2.5). The sister relationship of group erectoides to group arachis II received less than 50% BS support and did not have significant PP support, and could also be attributed to soft incongruence. Phylogenies based on the combined datasets resolved all major lineages and clades with strong to moderate support (Figure 2.7). Thus, discussion of phylogenetic relationship and systematic implication for *Arachis* species will focus on the phylogenies based on the combined dataset.

Basal Grade. Phylogenetic analyses of the combined ITS and *trn*T-*trn*F datasets produced trees that resolved *A. macedoi* of sec. *Extranervosae* as sister to remaining *Arachis* (Figure 2.5). The extended ITS dataset included three additional species of sec. *Extranervosae, A. burchellii, A. lutescens,* and *A. villosulicarpa,* which were resolved in one clade at the base. Galgaro et al. (1998) showed *A. burchellii* and *A. macedoi* appearing in one cluster in a RAPD based dendrogram. Gimenes et al. (2000) included these species in their UPGMA analyses of RFLP data and found that they formed a cluster with 81% BS support. These two studies support the placement of *A. burchellii* and *A. macedoi* in one lineage in this study. The grouping of *A. macedoi* with *A. lutescens* (94% BS-IN, 98% BS-IC, 1.00 PP-IN, 1.00 PP-IC) and *A. burchellii* with *A. villosulicarpa* (92% BS-IN, 98% BS-IC, 1.00 PP-IN, 1.00 PP-IC) in strongly-supported clades can be further bolstered with morphological characters (Krapovickas and Gregory, 1994). Both *A. macedoi* and *A. lutescens* possess leaflets that are smooth on the upper surface, while the leaflets of *A. burchellii* and *A. villosulicarpa* are covered in soft fine hairs. The phylogenetic position for sec. *Extranervosae* does not completely agree with the phylogenetic notions of Krapovickas and Gregory (1994) who considered sec. *Extranervosae* to be a "presumably older

section", sec. *Trierectoides* is "probably the most primitive [section] of the genus." They based their conclusion on the assumption that sec. *Trierectoides* is "genetically very isolated" from the remaining sections of the genus. In this study, the two species in sec. *Trierectoides* were nested deeper in the tree within group erectoides lineage (Figure 2.5).

The second diverging lineage, sec. *Triseminatae* (Figure 2.5), received 79% BS-IN, 68% BS-IC, and full PP support for their exclusion from remaining *Arachis* species. Krapovickas and Gregory (1994) considered this section to be one of the "older" sections in the genus because it is genetically isolated based on unsuccessful attempts to generate hybrids in crosses between sec. *Triseminatae* and representative species from other sections of the genus. The UPGMA analysis of microsatellite markers showed the accessions of *A. triseminata* forming a cluster distinct from the remaining species of the genus (Hoshino et al., 2006). The placement of the third diverging lineage, sec. *Caulorrhizae* (*A. pintoi* and *A. repens*) received full PP and BS support. The affinities between sections *Triseminatae* and *Caulorrhizae* have been previously demonstrated in the UPGMA analysis of RAPD and RFLP data (Galgaro et al., 1998). These two species of sec. *Caulorrhizae* are very closely related based on data from hybridization and pollen fertility, microsatellite data, RFLP, and isozymes (Gimenes et al., 2000; Gregory and Gregory, 1979; Hoshino et al., 2006; Palmieri et al., 2005). Morphologically, *A. pintoi* and *A. repens* are easily distinguishable on the basis of leaf shape and leaflet size (Gimenes et al., 2000; Krapovickas and Gregory, 1994) from other species of the genus.

Group erectoides. The next diverging lineage in the phylogeny based on the combined dataset is a heterogeneous lineage comprised of sections *Erectoides, Heteranthae*, *Procumbentes,* and *Trierectoides* (Figure 2.5). The structure of group erectoides in the analyses of the extended and

nonextended dataset were similar with the exception that the extended ITS dataset included *A. glabrata* from sec. *Rhizomatosae* (Figure 2.4). A similar grouping was recovered in UPGMA analysis of AFLP data (Gimenes et al., 2002b), with the exception that the *Trierectoides* species were not used in that study. However, their cluster received <50% BS support and its subgroups also tended to have low BS support. The exception was the sister relationship between *A. dardani* and *A. rigonii*, which was strongly supported (99% BS support) in the Gimenes et al. (2002) study. The *Arachis* phylogenies produced using combined ITS and trnT-*trn*F data resolved *A. rigonii* as sister to *A. dardani* and *A. appressipilia* (the latter was not included in the Gimenes study) with full PP and good BS support (78%) when indels were included as characters. Using microsatellite data, Hoshino et al. (2006) recovered a cluster of species from sections *Erectoides* and *Procumbentes* only. The emergence of erectoides in this study as a strongly supported lineage (1.00 PP-IN, PP-IC, 97% BS-IN, 99%BS-IC) can be explained in a historic perspective. Section *Trierectoides* was previously treated as part of sec. *Erectoides,* but was later segregated from this section based on the presence of three leaflets instead of the four leaflets found in all other sections (Krapovickas and Gregory, 1994). Species currently recognized as sec. *Procumbentes* were also historically included in sec. *Erectoides*, but were removed by Krapovickas and Gregory (1994) based on their decumbent habit and flower development along the length of the branches. Therefore, except for sec. *Heteranthae,* it is not surprising to see the other members converge into one lineage with such strong support.

Group arachis I. Members of sec. *Arachis* have appeared in one cluster in phenetic analyses (Gimenes et al., 2002b; Milla et al., 2005) and in a well supported lineage in phylogenetic analyses (Tallury et al., 2005), with compositions different from the arachis I and arachis II

recovered here. The species resolved in group arachis I included aneuploids, B genome, D genome, and one A genome species from sec. *Arachis,* in two subclades.

The fully supported aneuploids subclade was comprised of *A. palustris* and *A. praecox* (Figure 2.5). Inclusion of the ITS sequence from the third aneuploid, *A. decora,* in the extended dataset resolved all three aneuploids in the same subclade with full support from all analyses (Figure 2.4). These three aneuploid species were classified within sec. *Arachis* based on morphological features (Krapovickas and Gregory, 1994). Only a few studies focusing on sec. *Arachis* have included these aneuploid species (Bravo et al., 2006; Creste et al., 2005; Gimenes et al., 2007; Milla et al., 2005; Tallury et al., 2005). Microsatellite data placed accessions of these species together in a cluster with the A genome species *A. diogoi, A. kuhlmannii,* and *A. simpsonii* (Bravo et al., 2006). In AFLP based dendrograms, the aneuploids formed a separate cluster (Milla et al., 2005; Tallury et al., 2005). However, in the *trn*T-*trn*F study of Tallury et al. (2005) and here, the aneuploid species appeared within the B and D genome clade and were excluded from the A genome species with strong BS and PP support, implying higher genetic affinities to the B and D genomes than the A genome (Figures 2.3-2.5). The phylogenetic placement of the aneuploids points to chromosomal rearrangement following the split of arachis I and arachis II from a common ancestor. This event resulted in two sister lineages representing the aneuploids (*2n*=18) and the mostly B genome species that maintained the common chromosome complement of *2n*=20. This interpretation is more parsimonious than a loss of a pair of chromosomes in the aneuploids and their reappearance in the mostly B genome lineage.

The B genome species formed the second subclade of group arachis I along with the lone D genome species *A. glandulifera*. The B genome is identified by the lack of the small "A" chromosome characteristic of the A genome (Smartt et al. 1978; Fernández and Krapovickas,

1994; Krapovickas and Gregory, 1994). Within this subclade, B genome species resolved into two groupings based on the combined dataset (Figure 2.5). The first included *A. ipaensis, A. batizocoi,* and *A. cruziana,* while the second was comprised of *A. benensis, A. trinitensis, A. williamsii, A. magna, A. valida,* and the D genome species *A. glandulifera.* The emergence of *A. ipaensis* with *A. batizocoi* and *A. cruziana* in the phylogeny based on combined plastid and nuclear data was not expected. *Arachis ipaensis* has previously been considered more closely related to *A. magna* than to either *A. batizocoi* or *A. cruziana* based on data from crossability and RFLP (Burow et al., 2009), RAPD and RFLP (Gimenes et al., 2002a), AFLP (Milla et al., 2005), sequence of nuclear hypervariable regions (Moretzsohn et al., 2004), and microsatellite markers (Bravo et al., 2006). These studies suggests that species lacking the A chromosome, and thus identified as B genome species, or non-A genome species as suggested by Seijo et al. (2004), are a fairly heterogeneous group. Burow et al. (2009) had proposed the B genome be split into an *A. batizocoi* group and *A. ipaensis* group based on RFLP data. These proposed relationships are supported here only with the ITS data (Figure 2.3, 2.4)

Recently, Robledo and Seijo (2010) suggested that B genome, or non-A genome species, be separated into three genome groups based on heterochromatin detection and mapping of rDNA loci using FISH. The genome groups proposed were B (*A. ipaensis, A. gregoryi, A. magna, A. valida,* and *A. williamsii*), F (*A. benensis* and *A. trinitensis*), and K (*A. batizocoi, A. cruziana,* and *A. kropovickasii*). The proposed F genome species appear nested within arachis I with moderate BS (64% IN, 69% IC) and full PP support (Figure 2.5). The proposed K genome group (*A. batizocoi* and *A. cruziana*) formed a clade with full support in all analyses. The species in the proposed B genome group were not recovered as a single grouping in the combined and *trn*T-*trn*F data analyses but did so in the ITS data analyses (Figure 2.3, 2.5). This

may reflect the impact of the maternally inherited *trn*T-*trn*F on the evolutionary history of the group. In the *trn*T-*trn*F phylogeny, *A. ipaensis* resolves sister to the proposed K genome species. The placement of *A. ipaensis* was inconsistent depending on the genomic region used (Figure 2.3, 2.5). Tallury et al. (2005) recovered *A. ipaensis* as sister to *A. batizocoi* and *A. cruziana* in an ML network based on *trn*T-*trn*F sequence data, albeit with weak BS support. In the ITS based trees (Figures 2.3 and 2.4), species belonging to the proposed K group resolved in one group with moderate-to-low BS (61% IN, 67% IC) and significant PP support (0.97 IN, 0.97 IC). The F genome species appeared as a polytomy in the tree based on the ITS dataset.

Only one species in *Arachis*, *A. glandulifera,* has been designated to have the D genome (Stalker, 1991). The placement of this species in arachis I received strong support in the analyses of partitioned and combined data sets (Figures 2.3-2.5). However, its position within the clade differed based on the genomic region used. The ITS data showed low support for its placement as sister to *A. batizocoi* and *A. cruziana*, whereas the *trn*T-*trn*F data included it in the grouping of *A. magna, A. williamsii, A. benensis, A. trinitensis*, and *A. valida* with moderate BS and significant PP support (Fig. 2.4). It is to be noted that both the *trn*T-*trn*F and combined data showed *A. glandulifera* grouping with *A. valida,* but support varied (Figures 2.4, 2.5). Tallury et al. (2005) resolved a moderately supported lineage that included *A. glandulifera* with *A. williamsii* and *A. benensis*; *A. magna, A. valida,* and *A. trinitensis* were not included in that study. Therefore, although a common ancestry for the B and D genome species is evident, there seems to be disagreement in the placement of *A. glandulifera*, possibly caused by the mode of inheritance of the genomic regions or amount of phylogenetic signal.

Group arachis II. This group was comprised of species that have been identified mostly as A genome species, but also included one species from section *Erectoides, A. major* (Figures. 2.3-2.5). In the first study that covered representatives of several sections of *Arachis* (Gimenes et al., 2002b), sec. *Arachis* formed a distinct cluster in a UPGMA tree based on AFLP data that received less than 50% support. Lack of resolution is quite evident in arachis II clade in this study (Figures 2.3-2.5), as well as in previous studies (Gimenes et al., 2002b; Tallury et al., 2005). The low resolution and support among members of arachis II may imply recent origin or low rate of substitution.

*Origin of the allotetraploids in sec. Arachis***.** The differential inheritance of the ITS and *trn*T-*trn*F regions provided insight into the evolutionary history of the allotetraploids, *A. hypogaea* and *A. monticola*. The *trn*T-*trn*F analysis confirmed the maternal origin of the A genome since the allotetraploids appeared as part of the A genome lineage but in a polytomy (Figures 2.3, 2.5). The ITS clones further substantiated the results of the *trn*T-*trn*F data and provided evidence for the paternal origin of the B genome (Figs. 2.2-2.4) as previously noted by Kochert et al. (1991), Jung et al. (2003), and Seijo et al. (2004). Although the presence of polytomies prevented us from pinpointing the exact species donors, the putative donor species, *A. duranensis* and *A. ipaensis*, were recovered in those polytomies (Figures 2.3-2.5). Therefore, better resolution and support using more genomic regions with higher substitution rate is needed before molecular phylogenetics can provide support for the ancestry of the A and B genomes.

Systematic implication

At the present, the systematic monograph of Krapovickas and Gregory (1994) for the genus *Arachis* represents the only comprehensive treatment. In their intuitive treatment, they recognized nine sections based on morphological features from the leaf, hypocotyl, fruit, flower, and branching habit. The size of these sections varied from being monotypic, such as sec. *Triseminatae*, to considerably large ones, such as sec. *Arachis*. Krapovickas and Gregory (1994) also provided information on geographic distribution and polyploidy levels for these sections. This study is among the first molecular phylogenetic treatment for the genus using nuclear and plastid genomic sequences to examine the evolutionary history of species within all nine sections. Based on the phylogenetic trees generated, only two sections appear monophyletic, sec. *Extranervosae* and sec. *Caulorrhizae*, besides the monotypic sec. *Triseminatae* (Figure 2.3-2.5).

Krapovickas and Gregory (1994) recognized sec. *Extranervosae* on the basis of perennial habit, expanded corolla, and orange standard with red veins on its lower surface. The present study included one species for each of the partitioned and combined ITS and *trn*T-*trn*F datasets, while four of the nine species were used in the extended ITS dataset. It is evident from our extended ITS analyses that the section is monophyletic (Figure 2.4). Our results were consistent with those of Gimenes et al. (2002b), which placed *A. macedoi* and *A. burchellii* (the only two species used of sec. *Extranervosae*) in one cluster with high BS support, and with Galgaro et al. (1998) study where five of the species appeared in one cluster in their UPGMA analyses of RAPD and RFLP data. Thus based on the extended ITS data, we support the monophyly and thus taxonomic treatment of sec. *Extranervosae*.

Section *Triseminatae* is monotypic and it is characterized morphologically by the apomorphy of cotyledons with veins deeply sunken on the upper surface (Krapovickas and Gregory, 1994). In an attempt to generate intersectional hybrids, Gregory and Gregory (1979) did not succeed in obtaining hybrids between *A. triseminata* and members of other sections. Further, very few hybrids were obtained when crosses were made among various accessions of *A. triseminata*, indicating the presence of an intraspecific barrier within this species. The two accessions of *A. triseminata* (GK 12881 and GK 12922) used in this study appeared in one clade with full PP and BS support (Figures. 2.3-2.5), providing support for the monophyly of the species. Therefore, our study confirms the sectional status of *Triseminatae* and its isolated position.

Similarly, this study also confirmed the monophyletic nature of sec. *Caulorrhizae* with both *A. pintoi* and *A. repens* appearing in one clade in all analyses with high BS and full PP supports (Figs. 2.3-2.5). Crosses between *A. repens* and *A. pintoi* produced hybrids that had high pollen stainability (86.8%; Gregory and Gregory, 1979), implying very close genetic relationship. Similar conclusions have emerged from studies based on AFLP, microsatellite, and RAPD data (Gimenes et al., 2000; Hoshino et al., 2006; Palmieri et al., 2005). Section *Caulorrhizae* is characterized by procumbent branching habit, standard of the petals with red lines on its upper face, flowers and fruit that develop along the length of the branch, and stems that form roots at the nodes (Krapovickas and Gregory, 1994). Its two species can be separated morphologically on the basis of leaf shape and leaflet size (Gimenes et al., 2000; Krapovickas and Gregory, 1994). However, despite the differences in leaf morphology, the *trn*T-*trn*F and ITS sequences were identical. Similarly, protein profiles in the Bertozo and Valls (2001) study could not discriminate between these two species.

Since sec. *Heteranthae* (Am genome) is represented by one species, *A. dardani*, in this study; its taxonomic status cannot be assessed at this time. However, it appears to show phylogenetic affinities to sections *Erectoides* (E_2), *Procumbentes* (E_3), *Rhizomatosae* (R_2) and *Trierectoides* (E_1) as evident in the analyses of partitioned and combined datasets (Figures 2.3-2.5). Gimenes et al. (2002b) showed *A. dardani* and *A. rigonii* of sec. *Procumbentes* forming a cluster in their AFLP based UPGMA phenogram, in partial agreement with this study.

The majority of the species representing sections *Erectoides, Trierectoides,* and *Procumbentes* in our study were formerly placed in sec. *Erectoides* as series *Tetrafoliolatae, Trifoliolatae,* and *Procumbensae* (Gregory et al., 1973). However, Krapovickas and Gregory (1994) raised series *Procumbensae* and *Trifoliolatae* to sectional level, whereas series *Tetrafoliolatae* retained the sectional name *Erectoides*. Taxonomic treatment for these sections was based on plant habit, leaflet number, flower color, and hypocotyl characters (Krapovickas and Gregory, 1994). Sections *Erectoides* and *Procumbentes,* containing 14 and nine species, respectively, and are characterized by the tetrafoliate leaves, cylindrical hypocotyl and standard with red lines on the upper face. The two sections differ in their branching habit (erect for sec. *Erectoides* vs. procumbent for sec. *Procumbentes*) and flower position where they are distributed along the branches in sec. *Procumbentes* and found closer to the base (collar) in sec. *Erectoides*. Section *Trierectoides,* encompassing *A. guarantica* and *A. tuberosa,* is characterized by the trifoliate leaf and tuberiform hypocotyl. Our study resolved the three sections in a highly supported lineage in all analyses (Figures 2.4-2.5), implying a potential nucleus for a new taxonomic unit.

In a study examining intersectional relationships using crossability data, Gregory and Gregory (1979) produced hybrids among species from the three sections (*Erectoides,*

Procumbentes, and *Trierectoides*); however, most of these hybrids were highly or completely sterile as indicated by pollen stainability. Krapovickas and Gregory (1994) used these cytogenetic results to support the establishment of sections *Erectoides, Procumbentes* and *Trierectoides.* Hoshino et al. (2006) generated a dendrogram based on microsatellite data that showed species of sec. *Erectoides* and *Procumbentes* intermixed in a cluster separate from another cluster containing species of sec. *Trierectoides*, implying a close genetic relationship between sec. *Erectoides* and *Procumbentes*. Raina and Mukai (1999) have noted in their FISH study of ribosomal DNA that species of sec. *Erectoides* and *Procumbentes* possess the same genome but belong to different sub-genomes as identified by Gregory and Gregory (1979).

The placement of one *A. major* accession within erectoides, while the other accession is in arachis II, was not unexpected (Figures 2.3-2.5). Krapovickas and Gregory (1994) had noted that crosses among *A. major* accessions from the furthest points of its distribution did not produce fertile hybrids. Thus, they suggested that although the accessions appear to be morphologically similar, it was possible that they belong to different biological species. Therefore, *A. major* is in need of a detailed study at the population level to determine species boundaries and its phylogenetic placement in the genus.

Section *Arachis* is defined morphologically on the basis of the vertical peg and lack of rhizomes (Krapovickas and Gregory, 1994). However, their monograph for the genus did not further subdivide the section into the currently recognized genome groups A, B and D. Studies using molecular markers have shown that species with the A and B genomes form distinct groups (Burow et al., 2009; Gimenes et al., 2002a; Halward et al., 1991; Hilu and Stalker, 1995; Tallury et al., 2005). The sole D genome species *A. glandulifera* was shown to be more closely related to the B genome than to the A genome species (Tallury et al., 2005), a relationship

supported in this study (Figures 2.3-2.5). However, sec. *Arachis* as a whole was not consistently recovered as a single lineage and its monophyly lacked strong support in all analyses. Although, *trn*T-*trn*F did not recover sec. *Arachis* as a single lineage, the monophyly of it is well supported with the ITS and combined datasets. Further, we tentatively consider its two components groups arachis I and arachis II as two separate but closely related clades.

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CHAPTER 3: In search of a hypoallergenic peanut among wild relatives

Abstract

The peanut (*Arachis hypogaea*) is an economical and nutritious food, but it also has the ability to cause severe allergic reactions. Peanut allergies affect 1.2% of US population, whose only treatment for this immunological disease is strict avoidance. The majority of research on peanut allergens has focused on the crop. Only a few studies have been conducted on the putative progenitors of the crop, *A. duranensis* and *A. ipaensis*. The peanut genus *Arachis* is comprised of 80 wild species, many of which are considered valuable genetic resources for the crop. Twelve *Arachis* species were examined to identify potentially hypoallergenic orthologs of the peanut allergen Ara h 2. The major allergen Ara h 2 is a seed storage protein that is recognized by 90% of peanut-allergic individuals. Deduced protein sequences and homology models of Ara h 2 ortholog revealed that mutations were concentrated in a loop that connected α-helices H2 and H3. The differences in the H2-H3 loop included polymorphisms in immunodominant epitopes #6 and 7, as well as deletions to the epitopes' shared DPYSPS hexamer motif. B-cell epitope prediction programs ElliPro and DiscoTope were utilized to detect potential IgE-binding regions among the epitopes. Dot immunoblots of peptides representing H2-H3 demonstrated that variations among orthologs did have reduced antibody binding. The natural variations in Ara h 2 orthologs from the wild species demonstrate that wild *Arachis* species could be genetic resources in the development of a safer peanut crop.

Keywords: *Arachis*, Peanut allergy, Ara h 2 orthologs, homology modeling, wild species

Introduction

Peanuts (*Arachis hypogaea*) are an economical source of protein, vitamins and minerals. The peanut itself and its products are widely used throughout the food industry. In 2009, the US production of shelled peanuts was approximately 2.45 billion pounds (USDA-NASS, 2009). Despite their economic importance and nutritional value, allergies to peanuts have become a growing concern because of their wide use in the food industry and the severe reactions that it can elicit immunological reaction (Burks, 2003).

Food allergies are severe immune responses mediated by IgE to specific foods, usually proteins, and affect roughly 4% of the US population, or 12 million people (Sicherer and Sampson, 2006). The number of reported cases of children with food allergies increased 18% from 1997 to 2007 (Branum and Lukacs, 2008). A majority of food allergies (90%) are caused by proteins in a handful of foods, including cow's milk, shellfish, peanuts, tree nuts, hen's eggs, fish, wheat, and soybeans (Lehrere et al., 2002). Allergies to these food products have the potential to cause serious reactions, including anaphylaxis. The only treatment for food allergies is avoidance. Some children who have allergies to milk, eggs, soybean, and wheat can outgrow their allergies by the time they are old enough to go to elementary school (Wood, 2003). However, allergies to shellfish, tree nuts, fish, and peanuts are rarely, if ever, outgrown. In the US, allergies to peanut affect 1.2% of the population and the number of children with peanut allergies is growing (Sicherer et al., 2003).

To date, ten peanut allergens have been identified (Asero et al., 2002; Burks et al., 1992; Burks et al., 1991; Kleber-Janke et al., 1999; Mittag et al., 2004; Pons et al., 2002; Rabjohn et al., 1999). The majority of the peanut allergens are seed storage proteins, belonging to the two protein superfamilies to which most plant food allergens are classified (Breiteneder and Radauer,

2004), specifically, the cupin superfamily (vicilin - Ara h 1 and legumin - Ara h 3/4) and the prolamin superfamily (2S albumins - Ara h 2, Ara h 6, and Ara h 7).

The major allergen Ara h 2 is recognized by over 90% of patients who are peanut-sensitive (Koppelman et al., 2004). It comprises 6-9% of the total protein content in the seed (Koppelman et al., 2001). This allergen is present in two isoforms (Ara h 2.01 and Ara h 2.02), with an average mass of 17 kDa (Chatel et al., 2003). There are 10 IgE binding epitopes (Stanley et al., 1997), three of which are considered to be immunodominant. Ara h 2 has been predicted to be composed of two bundles of helices connected by a loop, which contains two of the three immunodominant epitopes (Barre et al., 2005; Lehmann et al., 2006). The tertiary structure is similar to 2S albumin proteins Ric c 3 (castor bean) and Ara h 6 (peanuts), both of which have been determined using NMR (Lehmann et al., 2006; Pantoja-Uceda et al., 2003).

Like other 2S albumins, it was predicted that Ara h 2 is comprised of five α-helices (Barre et al., 2005; Lehmann et al., 2006). Ara h 2 also has eight cysteine residues that are common to other proteins in the prolamin superfamily (Lehmann et al., 2006; Shewry et al., 2002): C-X_n-C-X-$_n$-CC-X_n-CXC-X-C-X_n-C. The α-helical structure and multiple disulfide bonds allow the allergen to maintain stability in high temperatures and resist digestion by proteases, allowing it to reach the gut immune system relatively intact (Moreno and Clemente, 2008). Another common feature of most 2S albumin proteins is the presence of a "hypervariable region" between helices 3 and 4 that contains the majority of the epitopes. Two of the three immunodominant IgE binding sites for Ara h 2, however, are located on the loop region connecting helices 2 and 3 (Lehmann et al., 2006).

The loop portions of proteins are important to protein function because they are exposed to the surface and can determine the functional specificity of a protein (Fiser et al., 2000). The

majority of variations, such as substitutions, insertions, and deletions, between members of a protein family are generally within loop regions. More information about these loops in allergens and how changes can affect them, in particular their ability to bind to IgE antibodies, could be useful for food allergy diagnosis and treatment (Moreno and Clemente, 2008).

Beyond the peanut crop species *A. hypogaea*, the allergen Ara h 2 has been examined only in the progenitors of this tetraploid species *A. duranensis* and *A. ipaensis* (Ramos et al., 2006; Ramos et al., 2008). The peanut genus contains 80 wild and cultivated species (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). These species have been grouped into nine sections, and the largest section *Arachis*, containing the crop and its progenitors species, has been divided into three genome groups: A, B, and D (Smartt et al., 1978; Stalker, 1991). The wild species of genus *Arachis* are considered to be important genetic resources for improving the crop (Rao et al., 2003).

In this paper, we examine the nucleotide, amino acid and structural differences of the Ara h 2 ortholog from wild species of *Arachis*. We show that the allergen orthologs from the various wild species *Arachis* contain significant changes in the loops that possess the epitopes with the DPYSPS motif. Also, species from sections *Caulorrhizae*, *Extranervosae,* and *Triseminatae* contain orthologs with mutations that could render them hypoallergenic. This is the first time that this serious allergen has been explored outside of the peanut crop and its progenitors.

Materials and Methods

Amplification of Ara h 2 orthologs

Genomic DNA was isolated from fresh leaf material of *Arachis* species (Table 3.1) following Milla et al. (2005). The allergen gene for *Ara h 2* was amplified by PCR using primers designed in our lab based on a sequence from *A. hypogaea* (GenBank accession AY007229).

The primer sequences were Arah2Start (5' ATGGCCAAGCTCACCATA 3') and Arah2-526 (5' AGCTTGCCTTAGTTAACACG 3'). Amplifications were conducted with 1x ThermoPol Buffer (New England Biolabs, Ipswich, MA), 200 μM dNTPs, 20 pmol of each primer and 1.5 U *Taq* DNA polymerase (New England Biolabs, Ipswich, MA) in 25 μl reactions. PCR amplifications were carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA) with 2 minutes of initial denaturing at 94° C, 25 cycles of 2 minutes denaturing at 94° C, 1 minute of primer annealing at 53° C, and 1 minute 30 seconds extension at 72°, followed by a final extension of 5 minutes at 72° C.

Table 3.1 Open reading frame length of *Ara h 2* orthologs from 12 wild species of *Arachis* belonging to eight sections. bp:basepair

Species	Section	Accession no.	ORF (bp)
A. batizocoi	*Arachis*	K 9484	498
A. duranensis		K 7988	483
A. glandulifera		KGSSc 30091	513
A. ipaensis		KGBSPSc 30076	519
A. palustris		VPmSv 13023	531
A. pintoi	*Caulorrhizae*	18747	477
A. paraguariensis	*Erectoides*	GKP 9646	468
A. macedoi	*Extranervosae*	GKP 10127	471
A. dardani	*Heteranthae*	GK 12943	468
A. rigonii	*Procumbentes*	10034	468
A. guarantica	*Trierectoides*		483
A. triseminata	*Triseminata*	GK 12922	477

Ara h 2 amplification products were resolved on 1% agarose-TAE gels and cleaned using QIAquick PCR purification or QIAquick Gel Extraction kits (Qiagen, Valencia, CA). Cycle sequencing reactions were performed using the ABI Big Dye Terminator Ready Reaction kit (Applied Biosystems Inc., Foster City, CA) following the manufacturer's protocol and then were electrophoresed on Applied Biosystems 373, 377 or 3100 automated sequencers (Applied

Biosystems Inc., Foster City, CA) at Virginia Bioinformatics Institute at Virginia Tech or at the DNA Analysis Facility at Duke University.

Sequence alignment and phylogenetic analysis

Amino acid sequences were translated directly from nucleotide sequences in PhyDE 0.995 (Müller et al., 2005). Both nucleotide and amino acid sequences were aligned manually using PhyDE 0.995. Phylogenetic analyses of Ara h 2 orthologs for the nucleotide and amino acid datasets were analyzed using maximum parsimony in PAUP*(Swofford, 2003), incorporating the parsimony ratchet algorithm (Nixon, 1999) via PRAP2 (Müller, 2007). Maximum Parsimony (MP) analyses were conducted using heuristic tree searches with TBR branch swapping with 1000 random addition sequence replicates. Strict consensus trees were generated for each data set. Supports for the clades were obtained by performing bootstrap (BS) (Felsenstein, 1985) searches with 1000 replicates and 10 random sequence replicates.

In silico protein structure characterization: secondary structure prediction and solvent accessibility

The Jpred 3 and PredictProtein servers were used for secondary structure prediction (Cole et al., 2008; Rost et al., 2004). Homologous sequence searches performed using the Jpred 3 server utilized BLAST and PSI-BLAST, and predictions of secondary structures were performed by Jnet (Cole et al., 2008). PredictProtein identified and aligned homologous results using the algorithms of BLAST, PSI-BLAST and MaxHom (Sander and Schneider, 1991). The MaxHom algorithm examines the database of homology-derived secondary structures of proteins (HSSP); (Schneider et al., 1997). The Jpred 3 server predicts secondary structure elements with 81.5%

accuracy (Cole et al., 2008). Two additional secondary structure predictors are available on the PredictProtein server, PROFsec and PHDsec. PROFsec has a 76% accuracy rate, while PHDsec has a 71% accuracy rate (Rost et al., 2004). Since PROFsec prediction software is more accurate in secondary structure, this study focused on prediction from this software in addition to those produced by Jpred 3.

PROFsec also predicted percent of residues that are accessible to the solvent (Rost et al., 2004). Residue accessibility to the solvent is one property that influences the propensity for a protein to be recognized as an allergenic protein (Kulkarni-Kale et al., 2005; Pomés, 2010).

Homology models of Ara h 2 orthologs

Homology models of Ara h 2 orthologs were generated in MODELLER (Sali and Blundell, 1993) using the allergen Ara h 6 structure (PDBID: 1q2wA) as a template. In MODELLER, five models were generated. The best model was chosen based on the lowest Modeller Objective Function score. Since the loop region of Ara h 2 orthologs is larger than that of the Ara h 6 protein, these loop regions were refined using the Modloop server (Fiser et al., 2000), which predicts the conformation of the loop regions independent of known structures, satisfies spatial restraints, and the conformation for the loop region is optimized to possess the lowest pseudo-energy score. The majority of loop conformation predictions have been performed on loops with lengths of 14 residues or less. However, predictions of medium-sized loops by ModLoop were as good as combinatorial methods (Jamroz and Kolinski, 2010). Assessments of models after loop refinement were conducted using ANOLEA (Melo and Feytmans, 1997), and Procheck (Morris et al., 1992) on the SWISSMODEL server (Arnold et al., 2005) and ProSA (Wiederstein and Sippl, 2007).

Epitope prediction

B-cell prediction programs have allowed for identification of areas that could potentially contain Immunoglobulin E (IgE)-binding epitopes (Pomés, 2010). The majority of the epitope prediction methods look for linear epitopes, which can be identified from the protein sequence. Discontinuous or conformational epitopes are those that are present in the tertiary structure of the protein by residues that are spatially close to each other. Barlow et al. (1986) and Van Regenmortel (1992) have estimated that >90% of B-cell epitopes are conformational epitopes. In absence of an X-ray or NMR crystal structure for allergens, computational methods have been developed to predict potential B-cell epitopes.

Two programs, DiscoTope and ElliPro, were used in this study to predict linear and conformational epitopes (Andersen et al., 2006; Ponomarenko et al., 2008). DiscoTope predictions combine computational methods proposed by Nishikawa and Ooi (1980) to measure the amino acids located at the protein surface. Additionally, the NACCESS program (Prescott et al., 2003) has been incorporated with DiscoTope to determine the residues that are accessible to the solvent. ElliPro predicts epitopes through the use of three algorithms. First, it determines the approximate ellipsoid shape of the protein (Taylor et al., 1983), second identifies residues that protrude beyond the protein ellipsoid and the protrusion index (PI), and, lastly, it clusters neighboring protruding residues based on the PI. The ElliPro method was modified from Thornton et al. (1986), which focused on identifying linear epitopes based on the PI for residues that were beyond the approximate ellipsoid shape. The score reported are the averaged PI values for the residues identified (Taylor et al., 1983).

Human sera

Seven peanut-allergic subjects (18-34 years old) and four non-allergic subjects (19-26) were recruited for this study. Each subject donated 10 ml of whole blood, from which sera were isolated by centrifugation at 3000 rpm for 10 min. Sera samples were stored at -80° C until use. This study was approved by the Institutional Review Board at Virginia Tech (IRB# 08-377) and informed written consent was obtained from each subject.

Dot immunoblots of epitope orthologs from wild Arachis conglutin proteins

Ten IgE-binding epitopes were identified on the Ara h 2 by Stanley et al. (1997); two of which contain a DYPSPS motif. From the derived amino acid sequences, 15-18 oligomer peptides were generated by GenScript (Piscataway, NJ) to represent the portions of the orthologs with this motif or variations of it (Table 3.2). Hydrated peptides were spotted directly onto Protan nitrocellulose membrane in a dilution series ranging from eight nanograms to one microgram, and allowed to dry. Dot immunoblots were incubated in 5% non-fat milk in Tris-buffered saline with 0.5% Tween-20 (TBS-T; pH 7.5) for one hour at room temperature with shaking. Nitrocellulose containing blots were incubated overnight at 4°C in either 1:6000 diluted chicken α-Ara h 2 antibody or in 1:20 diluted sera from peanut allergic (PA) individuals. Dotblots were washed three times with TBS-T for 10 min after antibody incubation. The dot immunoblots probed with chicken α-Ara h 2 antibody were subsequently incubated with rabbit anti-chicken IgG horseradish peroxidase (HRP)-conjugated antibodies (1:2500; Bethyl Laboratories Inc., Montgomery, TX) for one hour at room temperature. The immunoblots incubated with human sera were incubated with rabbit anti-human IgE HRP-conjugated antibodies (1:1000; Bethyl Laboratories Inc.). ECL Plus detection reagents were used for the

HRP-conjugated secondary antibodies for chemiluminescent detection with a Fuji LAS-300 camera. Differences in signal were determined by densitometry using the FUJIFILM Multi Gauge ver3.X.

Table 3.2 Peptide sequences in cultivated peanut and species and five wild species representing portion of the loop containing the immunodominant epitope motif DPYSPS and variations of that motif.

Species	Length (AA)	Sequence
A. hypogaea	15	RDPYSPSQDPYSPSP
A. batizocoi	15	RDPYSPSQDPYKQDP
A. glandulifera	15	DPDRQDPYSPSQDPD
A. guarantica	16	EQDPYRQDPYGPSPYG
A. rigonii	15	EQDPYGPSPYGPSPY
A. triseminata	15	QSPYSQDPYRQEPYE

Results

Nucleic acid and peptide sequences of orthologs from Ara h 2

The *Ara h 2* orthologs across the genus *Arachis* were 471 - 531 bp in length. Species from section *Arachis* tended to have larger coding regions for *Ara h 2* orthologs, ranging from 483 – 531 bp (Table 3.1), with the largest from the aneuploid species *A. palustris* (531 bp) and the shortest from the A genome species *A. duranensis.* Outside section *Arachis*, the nucleotide sequence length ranges from 468-477 bp in orthologs from *A. dardani* (sec. *Heteranthae*), *A. paraguariensis* (sec. *Erectoides*), and *A. rigonii* (sec. *Procumbentes*).

A total of ten gaps, ranging from 3-30 bp in length, were included in the matrix and resulted in an alignment that was 558 characters in length. When aligned, the mutations present in the first 177 and 319-558 characters nucleotides were substitutions. Both length mutations

and substitutions were present in positions 178-316. This variable region ranged from 47-100 nucleotides in the *Arachis* orthologs. In the Ara h 2 orthologs, 40-100% of the mutations present were located in the variable region.

Peptide sequences of the Ara h 2 orthologs were deduced directly from the genomic sequences and ranged from 155-176 residues (Table 3.2). Substitutions in nucleic acid sequence of Ara h 2 orthologs that were outside of the variable region resulted in no more than two missense amino acid changes in the peptide sequence. The length mutations observed in the nucleotide sequences did not result in any premature truncation of the proteins (Figure 3.1).

Ortholog sequences from section *Arachis* species had 90-94% sequence identity to the published conglutin sequences from the crop and its proposed progenitors (*A. duranensis* and *A. ipaensis*). Species outside of section *Arachis* had 81-91% sequence identity. When compared to the other peanut allergens from the prolamin superfamily, Ara h 6 and Ara h 7, sequence identity ranged from 49-60% and 46-50%, respectively (Table 3.2).

Phylogenetic relationships among Ara h 2 orthologs

We previously examined phylogenetic relationships among species and sections within genus *Arachis* using plastid and nuclear genomic sequences (Chapter 2), to which the phylogenies produced based on aligned Ara h 2 sequences will be compared. Nucleotide sequences of orthologous Ara h 2 ORF from cultivated and wild *Arachis* species were aligned into a matrix that included a total of 558 characters. Three most parsimonious trees of 109 steps resulted from the MP analysis. Strict consensus tree that is comparable to the phylogenies based on ITS and *trn*T-*trn*F data (Figure 3.2A). Ara h 2 orthologs from *A. macedoi* and *A. triseminata* (100% BS) emerged at the base in one lineage with 100% BS support and were sister to the

remaining taxa. Following the two basal species, *A. rigonii, A. guarantica, A. paraguariensis,* and *A. dardani* were resolved. This group received strong BS support (92%) and is comparable to the group erectoides lineage resolved in the phylogenies based on sequence data from nuclear and plastid regions presented in Chapter 2. Unlike the phylogenies produced with ITS and *trn*T-*trn*F data, the ortholog from *A. pintoi* was the next to diverge and resolved sister to those from section *Arachis.* However, the sister relationship between *A. pintoi* and section *Arachis* orthologs was weakly supported (53% BS). The orthologs from section *Arachis* resolved together with good support (89% BS). Despite the high sequence identity between Ara h 2 ortholog from *A. duranensis* and *Ara h 2.01* (Ramos et al., 2006), the sister relationship between these two species received moderate 65% BS support (Figure 3.2A). The sister relationship between *A. ipaensis* and *Ara h 2.02* received good support (80% BS).

Orthologs from Ara h 2 peptide sequences resulted in an alignment that included a total of 185 characters. MP analysis resulted in 51 trees with lengths of 45 steps. The strict consensus tree displayed a major polytomy (Figure 3.2B). Ara h 2 orthologs from group erectoides species, *A. dardani, A.guarantica, A. paraguariensis,* and *A. rigonii* appeared together in a polytomy with moderate (79%) BS support. Orthologs from *A. macedoi* and *A. triseminata* were also resolved together in one clade, but with low (60%) BS support.

Table 3.3 Characterization of Ara h 2 orthologs from wild species based on *in silico* methods. α-helical regions and disulfide bonds were identified in tertiary structures produced by the homology modeling program MODELLER. Percent of residues forming α-helical elements and accessible to solvent were predicted using the PROFsec program.

Species	Peptide	% Sequence Identity		Helical regions	Disulfide bonds	% residues forming α-helices	% residues accessible to solvent
		Ara h 6	Ara h 7				
A. batizocoi	165	54	45	H1:34-39, H2:44-53, H3:91-102, H4a:110-119, H4b:123-126, H5:130-145	[33,109], [45, 96], [97,145], [111,153]	53.99	72.12
A. duranensis	160	59	50	H1:34-39, H2:43-52, H3:85-97, H4a:105-114, H4b:118-121, H5:126-140	[33, 104], [45, 91], [92, 140], [106, 148]	51.92	71.15
A. glandulifera	170	52	43	H1:34-39, H2:44-53, H3:92-107, H4a:115-124, H4b:128-129, H5:136-150	[33, 114], [45, 101], [102, 150], [116, 158]	55.29	71.18
A. ipaensis	172	51	44	H1:34-38, H2:43-53, H3:97-109, H4a: 117-126, H4b:130-134, H5:138-152	[33, 116], [45, 103], [104, 152], [118,160]	51.92	71.15
A. palustris	176	49	43	H1:34-40, H2:44-53, H3:100-113, H4a:121-130, H4b:134-138, H5:142-156	[33,120], [45, 107], [108-120],[122, 164]	56.82	73.30
A. pintoi	158	57	50	H1:34-40, H2:44-53, H3:83-95, H4a:103-112, H4b:116-120, H5:124-138	[33, 102], [45, 89], [90, 138], [104, 146]	56.33	71.52
A. paraguariensis	155	57	49	H1:34-40, H2:44-52, H3:81-92, H4a:100-109, H4b:113-116, H5:120-135	[33, 99], [45, 86], [87, 135], [101, 143]	58.06	71.61
A. macedoi	156	60	48	H1:34-40, H2:44-54, H3:82-93, H4a:100-110, H4b:114-117, H5:122-	[33, 100], [45, 87], [88, 136], [102, 144]	56.41	71.15

				136			
A. dardani	155	56	50	H1:34-39, H2:43-53, H3:80-92, H4a:100-109, H4b:113-117, H5:121-135	[33, 99], [45, 86], [87, 135], [101, 143]	56.77	72.26
A. rigonii	155	57	50	H1:34-40, H2:44-53, H3:80-92, H4a:100-109, H4b5:113-116, H5:120-135	[33, 99], [45, 86], [87, 135], [101, 143]	56.13	70.32
A. guarantica	160	55	46	H1:34-40, H2:44-53, H3:85-97, H4a:105-114, H4b:118-121, H5:125-140	[33,148], [45, 140], [91-104], [92, 106]	53.75	70.00
A. triseminata	158	56	48	H1:34-39, H2:44-53, H3:83-95, H4a:103-112, H4b:116-120, H5:124-138	[33,102], [45, 89], [90, 138], [104, 146]	60.13	71.52

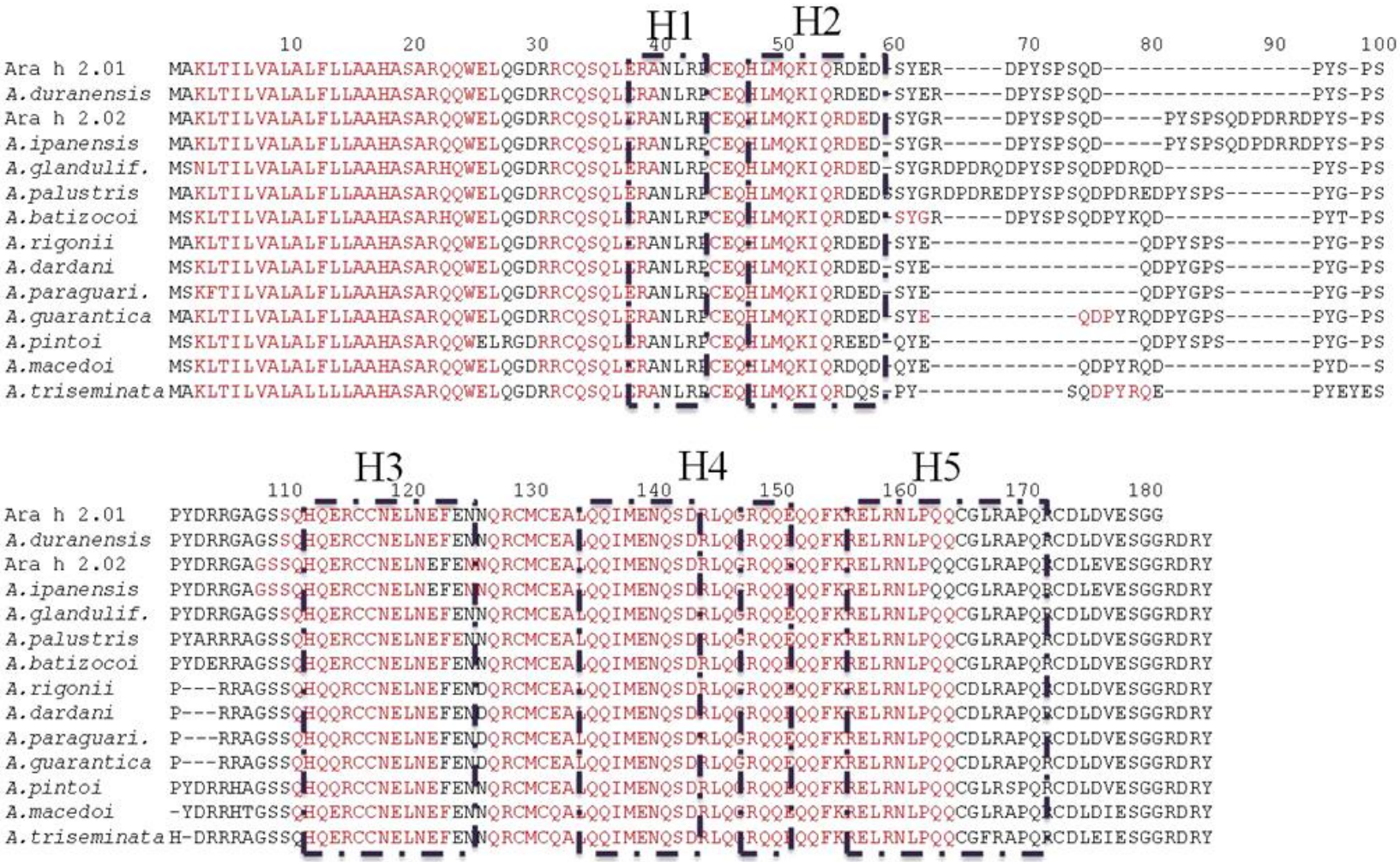

H1
H2
H3
H4
H5
Ara h 2.01
A.duranensis
Ara h 2.02
A.ipanensis
A.glandulif.
A.palustris
A.batizocoi
A.rigonii
A.dardani
A.paraguari.
A.guarantica
A.pintoi
A.macedoi
A.triseminata

Figure 3.1 Amino acid sequences of Ara h 2 orthologs from twelve wild *Arachis* species aligned with the two isoforms from the peanut crop (Ara h 2.01 and Ara h 2.02). Helical elements predicted by JPRED3 are highlighted in red. Jpred 3 predicts five helices. The secondary structural elements of conglutin protein were not affected by the length mutations within the protein. Helices identified in homology models of orthologs are also identified as H1-H5.

Secondary and tertiary structures among Ara h 2 orthologs

The allergen Ara h 2 belongs to the prolamin superfamily (Kreis et al., 1985), which consists of proteins that are predominantly comprised of α-helices connected by disordered loops. Consistent with members of their superfamily, Ara h 2 orthologs were predicted to be comprised of α-helices and disordered regions. The predicted amount of residues forming α-helices ranged from 51.92% in *A. ipaensis* to 60.13% in *A. triseminata* (Table 3.3). Secondary structure prediction of Ara h 2 orthologs by the Jpred 3 server identified five potential α-helices (Figure 3.1). PROFsec predicted similar helical regions among Ara h 2 orthologs from wild species (Appendix Fig C.1).

Homology models of Ara h 2 orthologs from wild species of the genus *Arachis* consisted of five α-helices, identified as H1-H5, connected by extended loops and arranged in a right-handed superhelix (Figures 3.3). The models produced here were consistent with Ara h 2 models previously reported (Barre et al., 2005; Lehmann et al., 2006). The models were also consistent with secondary structures predicted by Jpred3 and PROFsec, with the exception that the first helix (Figure 3.1) predicted to form between residues 2-27 remained a disordered region in the models produced by MODELLER.

Members of the prolamin family are stabilized by eight to ten cysteine residues that form intrachain disulfide bonds (Shewry et al., 1995). The template Ara h 6 structure determined by NMR contained ten cystine residues (Lehmann et al., 2006). However, eight cysteines were present in models of the Ara h 2 orthologs from wild *Arachis* species resulting in four disulfide bonds were identified in (Table 3.3).

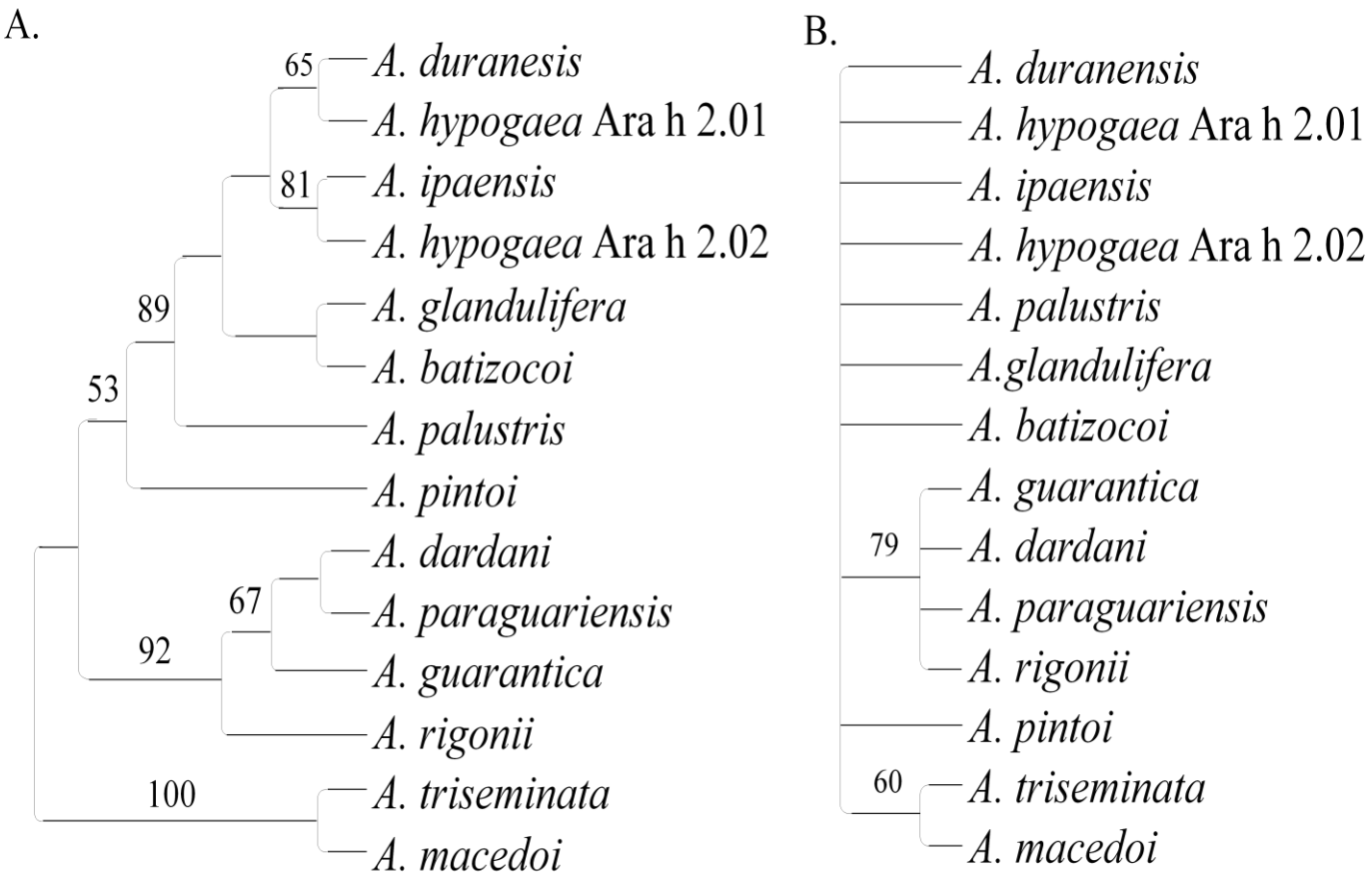


Figure 3.2 Strict consensus trees based on (A) nucleotide and (B) peptide sequences analyzed using MP. **A.** For the aligned Ara h 2nucleotide sequences, 81 of the total 558 characters were variable (14.5%), of which 41 were parsimony informative (50.6%). Three most parsimonious trees of 109 steps resulted from the MP analysis with the CI and RI values of 0.798 and 0.796, respectively. **B.** The peptide sequences consisted of 185 characters when aligned, of which 31 were variable (16.8%) and 15 were parsimony informative (48.4%). MP analyses resulted in 51 most parsimonious trees with lengths of 45 steps. The CI and RI were 0.800 and 0.763, respectively.

The majority of variations observed in the alignment corresponded to a loop spanning helices H2 and H3, mostly as length variations. In the template structure, Ara h 6, the loop between H2 and H3 was 17 residues in length (Lehmann et al., 2006). Though the H2-H3 loops in Ara h 2 ortholog structures were 1.59-2.76 times the size of the one found in Ara h 6. The shorter loops among the orthologs were predicted in group erectoides species *A. dardani*, *A. rigonii,* and *A. paraguariensis,* spanning 27-29 residues. Longer loops were predicted in *A. batizocoi, A. glandulifera, A. ipaensis* and *A. palustris*, with lengths spanning 38-47 residues. The conformation of the loops varied among the orthologs. The H2-H3 loop in the allergen Ara h 2.01, which is also identical to the *A. duranensis* ortholog, was extended (Figure 3.3, 3.4). The loops from the remaining orthologs appeared to be folded in towards the helices of the protein (Figure 3.4). The folded H2-H3 loop was more prevalent in *A. batizocoi*, *A. glandulifera*, *A. ipaensis,* and *A. palustris.*

Variations in amino acid composition of the H2-H3 loop were also present in Ara h 2 orthologs from wild *Arachis* species. Comparison of models to NMR and X-ray determined structures of 2S albumins in PDB showed that loops between helices H2-H3 were unique to these orthologs. Also, this loop possesses the immunodominant Ara h 2 epitopes 6 and 7. These two epitopes possess a hexapeptide DPYSPS motif (Stanley et al., 1997). Ara h 2 orthologs from section *Arachis* species tended to maintain the DPYSPS motif. An S-to-T variation at position 78 was present in the ortholog from *A. batizocoi* (Figure 3.4). The Ara h 2 ortholog from D genome species *A. glandulifera* lacked epitope 6; however, it contained an additional copy of epitope 7. The aneuploid species contained an R to E variation in a modified epitope very similar to epitope 6 and is identified here as epitope "6." *Arachis pintoi,* which resolved

sister to section *Arachis* species, contained an H2-H3 loop that possessed the epitope 7 and a part of the hexapeptide motif (PYGPS) that included an S to G variation at position 71.

Like *A. pintoi,* the same variations for the epitopes were also identified in *A. rigonii.* Wild species *A. dardani* and *A. paraguariensis* both possessed a variation of epitope 7 that contained an S-to-G variation at position 66, which is named here as epitope "7." *Arachis guarantica* ortholog loop included an epitope "7" and also two partial hexapeptide motifs (QDPY and PYGPS). The two orthologs from *A. macedoi* and *A. triseminata,* which grouped together at the base MP nucleotide-based tree, contained only the QDPY partial motif in the H2-H3 loop. *Arachis macedoi* possessed two QDPY portions of the hexapeptide motif. Assessments of Ara h 2 ortholog models were conducted on the SWISS-MODEL (Arnold et al., 2005) and ProSA servers (Wiederstein and Sippl, 2007). Ramachandran plots generated by PROCHECK showed that phi-psi angle combinations for residues of Ara h 2 orthologs were >90% favorable to generously allowed (Supplemental Table B.1). Energy profiles generated by ANOELA tended to identify residues located within the N terminus, H2, H4, and the C-terminus as high energy zones (Supplemental Figures A.1-12). Some residues within the H2-H3 loop also were identified as high energy zones. ProSA overall model quality z-scores for the Ara h 2 were similar to those obtained by NMR-determined structures, and its local model quality tended to have negative knowledge-based energy with the exception of the unstructured N- and C-terminal regions (Supplemental Table A. 2 and Figures A.13-24). Predictions in the C-terminus the majority of the Ara h 2 orthologs tended to receive the lowest scores, ranging from 0.516-0.675. The orthologs from *A. hypogaea* and *A. batizocoi* were not predicted to contain an epitope in the C-terminus.

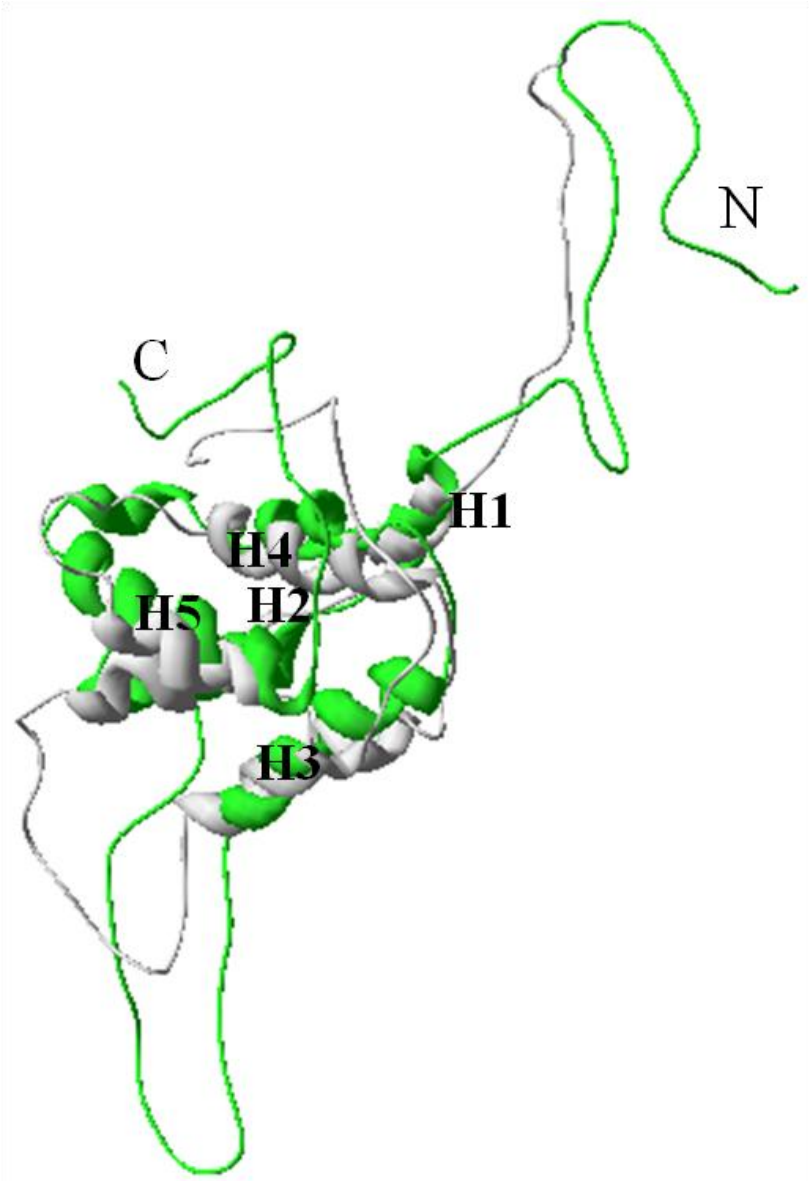
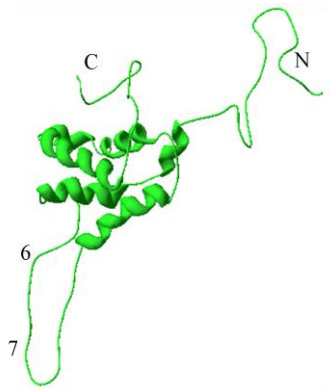
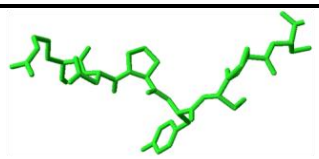
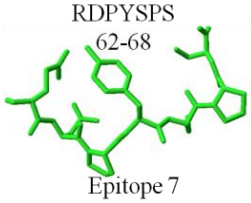
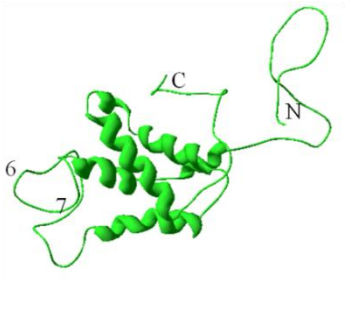
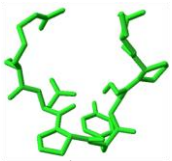
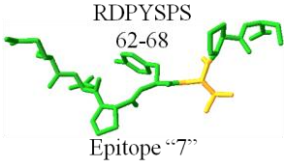
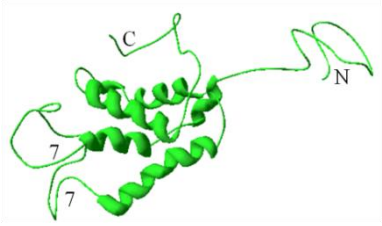
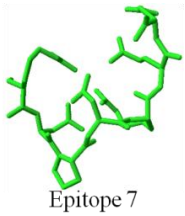


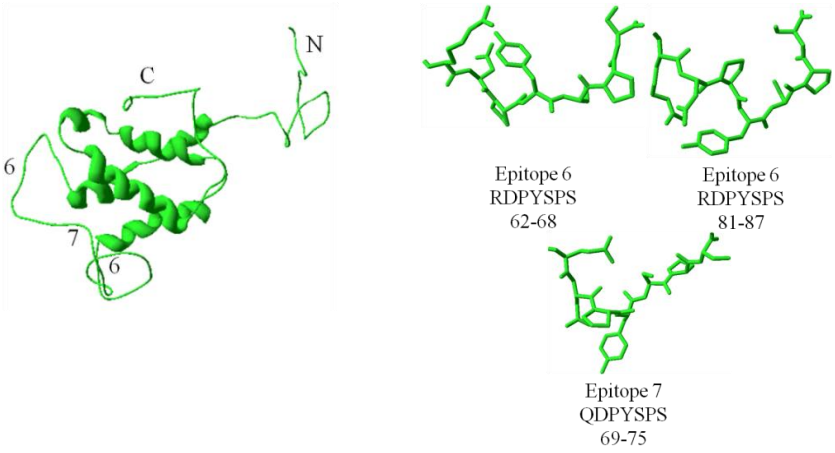
Figure 3.3 Model of Ara h 2 ortholog from *Arachis duranensis* (green) compared to the Ara h 6 template (PDB ID 1W2Q; gray). Helices are noted as H1-H5. N and C termini are also noted.

Antibody binding to peptides representing part of loop region within Ara h 2 orthologs

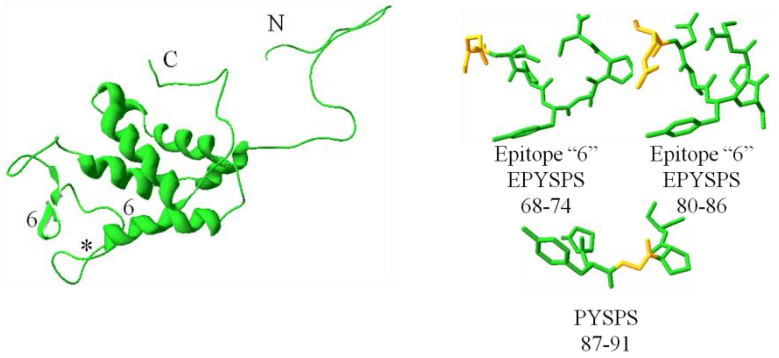
Peptides representing portions of the H2-H3 loop that contained variations at the nucleotide and amino acid levels were generated and antibody binding to these regions was assessed using dot immunoblots that were probed with sera from self-reported peanut-allergic individuals. The dot immunoblot probed with sera from PA-5 is shown (Figure 3.6A). Immunoblots probed with other sera showed similar results (not shown). Differences in antibody binding to the peptides representing loop portions from wild *Arachis* species orthologs were noticeable, and decreasing recognition corresponded with the lower amounts of peptides and protein applied. Variation in signal was most noticeable in the 40 ng row, which was analyzed semi-quantitatively using densitometry. When the background was subtracted from the densitometry reading, three categories of antibody recognition, high, moderate, and low, were apparent when comparing signal intensities to the peptide representing the loop from *A. hypogaea*. Peptides with binding of 50% or less compared to the peptide representing the H2-H3 loop *A. hypogaea* were considered to possess low antibody binding affinity (Figure 3.6B). Peptides with binding affinity of 50-75% were considered to have intermediate and 75% and above had high binding affinity.

Species	Orthologs Structure	Potential Epitopes
A. duranensis/ Ara h 2.01 (*Arachis*)		 Epitope 6 RDPYSPS 62-68  Epitope 7 QDPYSPS 69-75
A. batizocoi (*Arachis*)		 Epitope 6 RDPYSPS 62-68  Epitope "7" QDPYTPS 62-68
A. glandulifera (*Arachis*)		 Epitope 7 QDPYSPS 62-68  Epitope 7 QDPYSPS 79-85

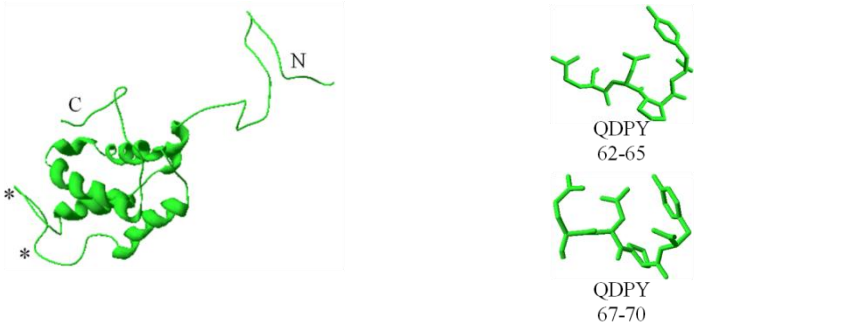
A. ipaensis (*Arachis*)



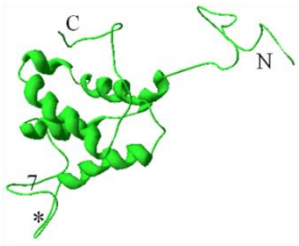
A. palustris (*Arachis*)

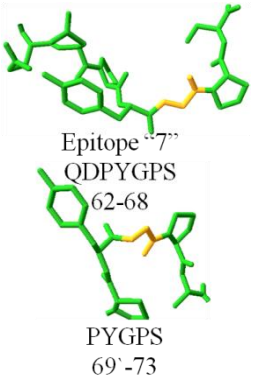


A. macedoi (*Extranervosae*)

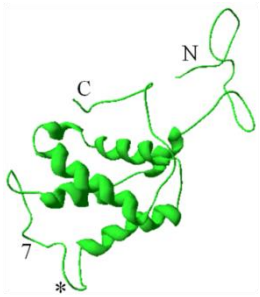


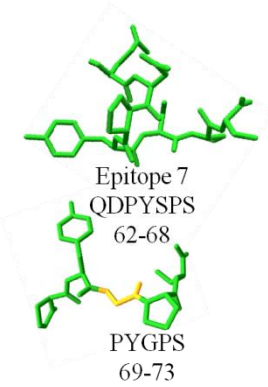
A. dardani (*Heteranthae*)



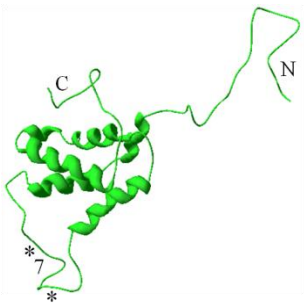


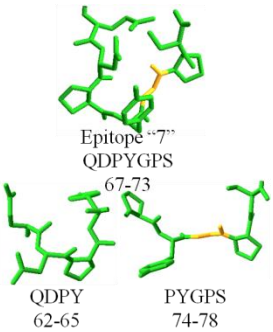
A. rigonii (*Procumbentes*)



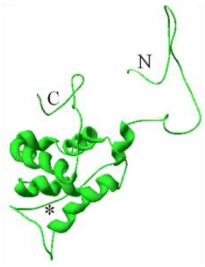


A. guarantica (*Trierectoides*)





A. triseminata
(*Triseminatae*)



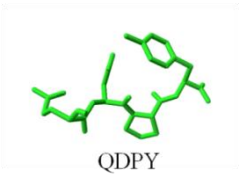


Figure 3.4 Homology models of Ara h 2 orthologs from section *Arachis* species generated by Modeller 9v6. The NMR structure of the peanut conglutin protein Ara h 6 (PDB 1w2q; (Pantoja-Uceda et al., 2003) served as a template from which the models were generated. Epitopes 6 and 7 initially identified in the Ara h 2 allergen from the crop are noted on the models and their conformations are also noted. Amino acid substitutions present in the epitopes among the orthologs are highlighted in yellow.

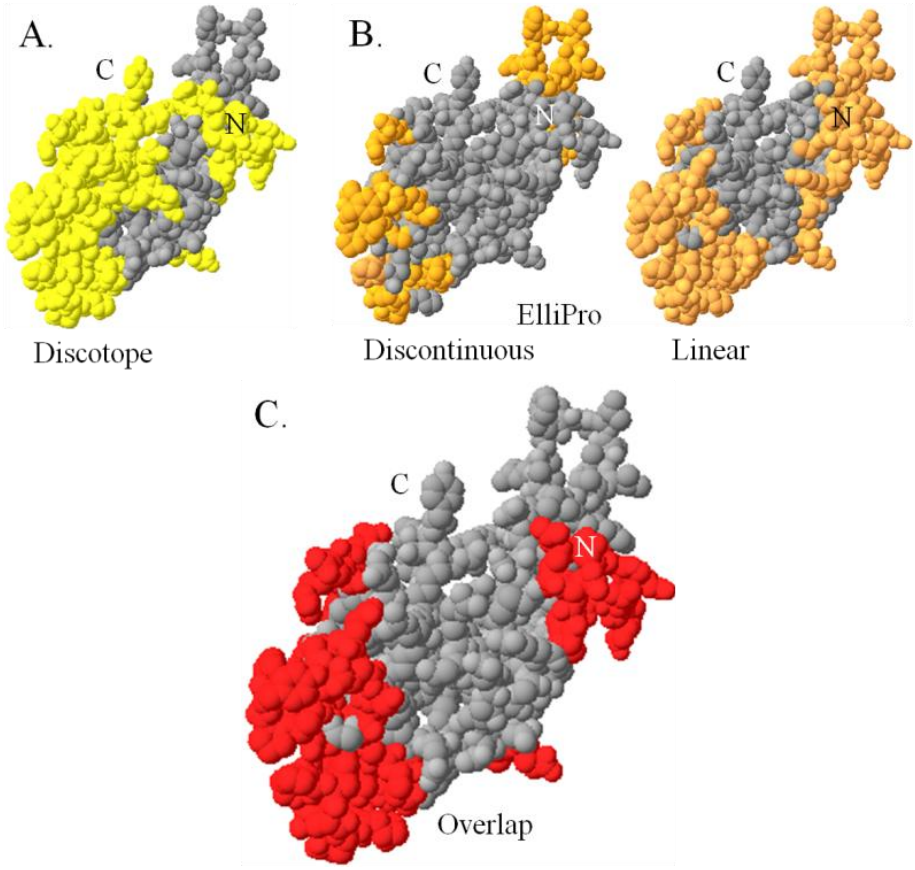


Figure 3.5 Predicted epitopes for Ara h 2 ortholog from *A. batizocoi*. **A.** Residues predicted to be potential IgE binding sites by the program DiscoTope on the surface of the allergen ortholog are shaded in yellow. **B.** Residues predicted to potential conformational epitopes by the program ElliPro are shaded in orange. **C.** Residues that were predicted as potential epitopes by both programs are shaded in red. Both programs predict the N-terminus and H2-H3 loop to be sites of potential antibody binding.

As expected, crude protein extract (CPE) from *A. hypogaea*, isolated Ara h 2, and the peptide representing the part of the loop from the *A. hypogaea* ortholog were highly recognized and signal was highest (Figure 3.6B). The peptides from *A. batizocoi* and *A. rigonii* orthologs were also highly recognized when compared to the one from *A. hypogaea*. The peptide representing the part of the loop ortholog from *A. glandulifera* was moderately recognized when compared to the one representing the crop. The lowest signals were produced by peptides representing portion of the loop from *A. guarantica* and *A. triseminata*. The signal from the *A. guarantica* peptide was 50.9% less than the one representing *A. hypogaea.* The peptide from *A. triseminata* had the lowest signal overall, 69.6% less when compared to the one from the crop.

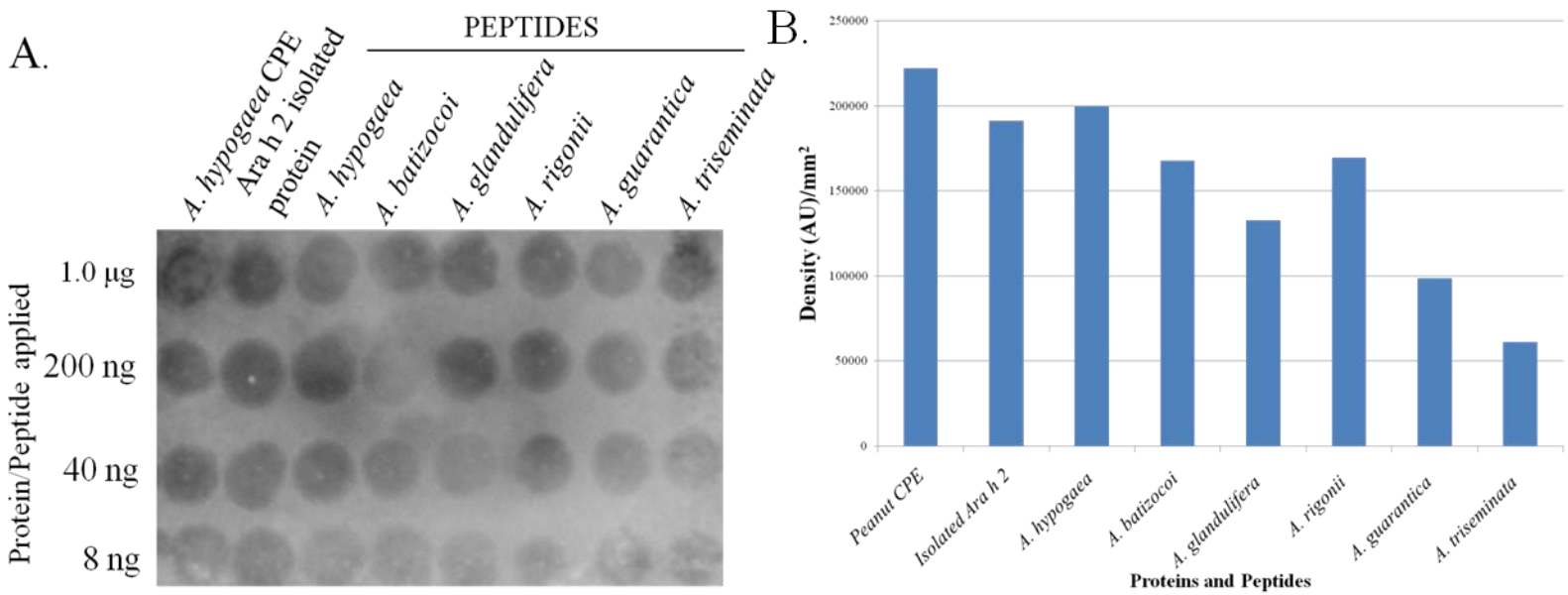


Figure 3.6 A. Dot Immunoblot of Ara h 2 ortholog peptides. Sera from peanut-allergic individual PA-5 was used to test the peptides representing portions of the loop region belong to orthologs from five wild species. Peptides were applied to the nitrocellulose membrane, with amounts decreasing by five-fold, then probed with sera. Densitometry measurements showed overall reduction in antibody binding to 40 ng of peptides from wild species, with signal from background subtracted. Greatest reduction in antibody binding was observed in peptides representing the orthologs from *A. guarantica* (50.6%) and *A. triseminata* (69.6%).

Discussion

Until now, the allergen Ara h 2 had been studied within the crop species and its putative progenitors (Ramos et al., 2006; Ramos et al., 2008; Stanley et al., 1997). Here we expanded the number of species used to include more species from *Arachis* genomes and additional other sections in the search for hypoallergenic proteins among members of genus *Arachis*.

Generally, Ara h 2 orthologs that were from species classified within section *Arachis* tended to be larger in length (Figure 3.3). When plotted on the phylogenetic tree from chapter one, the open reading frames and their subsequent products shows in size moving up the evolutionary tree. The *Ara h 2* ortholog gene tree was very similar to the *Arachis* phylogenies based ITS and combined ITS and *trn*T-*trn*F datasets (Figure 3.2). The notable deviation from the phylogeny is the placement of *A. pintoi* from sec. *Caulorrhizae*, which appeared in between the section *Arachis* orthologs and those from section *Erectoides.*

The structure of 2S albumins consists of five α-helices, which form two subunits, connected by disordered loops (Shewry et al., 2002; Shewry et al., 1995). The α-helices H1 and H2 appear to correspond to the light chain while H3 to H5 correspond to the heavy chain. The overall structure Ara h 2 from the crop was consistent with previous models (Barre et al., 2005; Lehmann et al., 2006). Homology models of the Ara h 2 orthologs from wild species were similar in structure to each other and to the model from the crop (Figure 3.4). The main difference between the homology modeling and the secondary structure predictions from Jpred 3 and PROFsec was the lack of structure at the N terminus. However, this lack in structure was due to the lack of structured regions along the N terminus in the template structure, Ara h 6 (PDB ID: 1W2Q). Additionally, the template also lacked a corresponding loop between H2-H3. Energy minimization refinement by MODLOOP allowed for residues within H2-H3 to possess

mostly favorable energy scores in ANOLEA and the local model quality plot in ProSA (Supplemental Figures A.1-A.12). The conformation of the H2-H3 loop for Ara h 2 orthologs varied from being fully extended (e.g. *A. hypogaea*/*A. duranensis*), kinked (e.g. *A. guarantica*), and being folded in towards the heavy and light chains (e.g. *A. batizocoi*; Figure 3.4). Unfavorable energy scores were apparent for residue located in the N-terminus; α-helices H2, H4, and H5; and portions of the C-terminus. Residues that received a high energy score in ANOLEA have been generally thought to have errors or interact with other regions (Melo et al., 1997).

Members of the prolamin superfamily contain a backbone structure of C -X_n-C-X-$_n$-CC-X_n-CXC-X-C-X_n-C (Shewry et al., 2002). While the number of cysteines varies from eight to ten within the protein sequence, these residues form four to five disulfide bonds that stabilize the protein and connect the heavy and light chains. The template structure, Ara h 6, possessed ten cysteines within its amino acid sequence. In the Ara h 2 orthologs from wild species, eight cysteines were present in the amino acid sequence, which were predicted to form four disulfide bonds in the homology models. Lehmann et al. (2006) speculated that the lack of the fifth disulfide bond allowed the C-terminus to lack a secondary structure and remain flexible.

The majority of the variations were located within the loop that connected helices H2 and H3. Typically, the hypervariable region in 2S albumin lies in the loop connecting H3 and H4 (Monero and Clemente, 2008). Variations in the H2-H3 loop appear to be specific to the Ara h 2 orthologs when compared to other 2S albums. The evolutionary trend appeared to be that the larger H2-H3 loops were present in the more derived species.

The changes located within the H2-H3 loop would affect two of the ten IgE-binding regions present in Ara h 2, epitopes 6 and 7. These two epitopes have been recognized as

immunodominant as they were consistently recognized by patient sera (Stanley et al., 1997). Both epitopes 6 and 7 share a hexapeptide motif, DPYSPS. The differences within the H2-H3 loop among Ara h 2 orthologs included additional residues between the epitopes and amino acid variations within the hexapeptide motif, and partial or complete deletions of either epitope (Figure 3. 4). Chatel et al. (2003) identified that the Ara h 2.02 isoform contains an H2-H3 loop that is 12 amino acids longer than Ara h 2.01, which was hypothesized could elicit a stronger reaction. The Ara h 2.02 isoform is an identical sequence to the Ara h 2 ortholog from *A. ipaensis* (Figure 3.1), its putative B genome progenitor. Based on the prediction by Chatel et al. (2003), one could reason that the ortholog from *A. palustris* could also elicit a stronger reaction because its H2-H3 loop is 16 amino acids longer than the Ara h 2.01 isoform and contains potential IgE-binding regions that resemble epitope 6. The difference between epitope 6 (DPYSPS) and the epitope "6" (EDPYSPS; underlined identifies the substitution) is the missense variation in which there is a slightly bulkier side chain. The ortholog from *A. batizocoi* contains a variation in epitope 7 that was also identified by Ramos et al. (2008) in an accession of *A. duranensis* (DPYSPS/DPYTPS). The S to T mutation had significant decrease in antibody binding. In addition to an epitope that contains a similar variation identified by Ramos et al. (2008), the loop was five residues longer than the one for Ara h 2.01. The Ara h 2 ortholog from *A. batizocoi* has the potential to be less allergenic than the orthologs from *A. palustris, A. duranensis*, and *A. glandulifera* as it contains a similar variation that was present in the *A. duranensis* ortholog identified by Ramos et al. (2008).

In the majority of the orthologs from species outside section *Arachis,* the H2-H3 loop tended to be shorter in length and have variations within a full or truncated epitope (QDPYGPS, PYGPS, PYSPS, or QDPY). *Arachis paraguariensis, A. guarantica, A. rigonii,* and *A. pintoi*

contained an S to G variation in potential epitopes, a polar charged residue to a hydrophobic residue (Figure 3.4). King et al. (2005) induced a similar variation by site directed mutagenesis (S to A), which resulted in a reduction in antibody binding to the recombinant protein product. Orthologs with a similar variation could potentially have reduced antibody-binding affinity. In the orthologs from *A. triseminata* and *A. macedoi*, a truncated version of the epitope (QDPY) was present (Figure 3.4). In combination with the shorter H2-H3 loop, these two species have the potential to be hypoallergenic.

The theoretical structure of the Ara h 2 orthologs allowed for linear and conformational B cell epitopes to be predicted by the programs ElliPro and DiscoTope. Predictions of epitopes would allow for better understanding of which residues would interact with an antibody and could aid in the design of an altered protein that could be used for immunotherapy (Pomés, 2010). Generally, epitopes are determined experimentally using overlapping peptides or protein fragments. For the peanut allergen Ara h 2, Stanley et al. (1997) determined 10 linear epitopes to be present among 61 residues (38.13%) using overlapping peptides. The linear epitopes predicted by ElliPro and conformational DiscoTope epitope predictions tended to identify a larger portion of the Ara h 2.01 to be potential epitopes, 48.5 and 58.8% respectively. DiscoTope predictions were set at a threshold score of -7.7, which allowed for 25% of the predicted residues to be false-positively considered to be epitopes (Andersen et al., 2006). Conformational ElliPro predictions predicted fewer residues as potential epitopes than the number determined experimentally (28.8%). Though the number of residues in the conformational epitopes as predicted by ElliPro was fewer than the other methods, these residues tended to receive higher protrusion index scores the further away they were spatial to the heavy and light chains. While these programs tended to predict more residues as epitopes than those

determined experimentally, they could aid in identifying which regions of a protein should be focused on for development of hypoallergenic or immunothearpy targets.

Predicted epitopes were influenced by the conformation of ortholog models based on the Ara h 6 template. The H2-H3 loop conformation varied from being extended, as seen in the *A. hypogaea* Ara h 2.01/*A. duranensis* model, or folded in towards the α-helices, as seen in the Ara h 2 orthologs from wild species (Figure 3.4). The linear epitope prediction for *A. hypogaea* 2.01/*A. duranensis* model by ElliPro gave H2-H3 residues 54-59 a lower protrusion score (0.605) than residues 64-83 (0.77), as residues 54-59 were closer to the helical core of the modeled protein. The ElliPro conformation epitope prediction gave the highest protrusion index score (0.975) to five residues that were furthest away from the light and heavy chains. The remaining residues that comprised the H2-H3 loop were also recognized as conformational epitopes, but with lower protrusion index scores (0.506-0.781). Both programs did include the residues of immunodominant epitopes as part of their predictions in *A. hypogaea* 2.01/*A. duranensis* ortholog.

For the Ara h 2 orthologs from wild *Arachis* species, the H2-H3 loop was consistently predicted as a conformational epitope by DiscoTope in all of the Ara h 2 orthologs (Supplemental Table C.2), even when the loop was folded in towards the α-helices (Figure 3.4). Amino acid residues received higher protrusion index scores from ElliPro the further away it was located from the heavy and light chains (Supplemental Table C.1). Overall, residues that were identified as potential epitopes received scores that ranged as low as 0.545 to 0.934. Amino acid variations seen in the peptide sequence or the H2-H3 loop were not reflected in the epitopes predicted in orthologs from wild species.

Interestingly, conformational epitopes that included the residues of the H2-H3 loop and those of helices H4 through H5 were identified here (Figure 3.5 and Supplemental Table B2). These potential conformational epitopes have not been previously identified. ElliPro conformational predictions did recognize these residues as part of an epitope in the orthologs from *A. ipaensis* (R55, D56, E57, Y60, G61, R62, D63, P64, Y65, R135, Q137), *A. guarantica* (D56, E57, R119), and *A. triseminata* (R55, D56, S58, P59, Y60, R117, G120, R121). Molecular dynamic simulations for the Ara h 2 models were conducted by W.J. Allen in the Bevan lab (Department of Biochemistry, Virginia Tech). The MD simulations showed that the residues located between helices H4 through H5 were determined to be very flexible based on root mean square fluctuation (RMSF) measurements. The RMSF measurements suggest that residues between H4 and H5 could be a functional area and in combination of the data from DiscoTope and ElliPro, this region with the H2-H3 loop could serve as a conformational epitope in Ara h 2 orthologs.

In addition to the computational predictions of epitopes, peptides representing the first 15-16 residues of the H2-H3 loop from *A. hypogaea* and five wild *Arachis* species were synthesized and assessed for antibody binding using dot immunoblots. The peptide representing the loop from *A. hypogaea* produced higher signal than the isolated Ara h 2 protein (Figure 3.6). Of the linear epitopes identified by Stanley et al. (1997), epitopes 6 and 7 were consistently recognized by patient sera. In comparison to isolated Ara h 2 protein, epitope 6 was more prevalent in the *A. hypogaea* peptide spots than the isolated Ara h 2 spots (Figure 3.6). Peptides representing the H2-H3 loop from *A. batizocoi* (section *Arachis*) and *A. rigonii* (section *Procumbentes*) had the next highest antibody affinity, as indicated by the densitometry reading. Even though there were variations in the peptides representing these two species, each peptide

contained the DPYSPS motif (Table 3.2). Despite a complete hexamer present in the sequence for the peptide representing the *A. glandulifera* (section *Arachis*) H2-H3 loop, this peptide had lower antibody binding affinity as shown in the densitometry measurement (Figure 3.6), which was 32.6% less than the measurement of the peptide from the H2-H3 loop from *A. hypogaea*. While the *A. glandulifera* peptide contains a DPYSPS motif, it also appears to possess fewer residues with bulky side chains (Table 3.2). The peptide from *A. guarantica* had next to the lowest antibody binding affinity, with densitometry measurements that were 50.6% less than the peptide from *A. hypogaea* (Figure 3.6). The peptide representing the H2-H3 loop from *A. guarantica* possessed a variation within the hexameric motif (DPYSYS/DPYGPS). The peptide from *A. triseminata* (section *Triseminatae*) had the lowest antibody affinity. Densitometry measurement for peptide was 69.4% less than the peptide from *A. hypogaea.* It also lacked the whole DPYSPS motif within the peptide sequence. Only the DPY portion of the motif was present in the peptide sequence for *A. triseminata*, suggesting the lack of a full DPYSPS motif played a role in reduced antibody binding affinity.

In addition to the completeness of the DPYSPS motif, the number of prolines and glutamines appears to have some correlation to antibody binding and subsequent intensity. The amount of proline residues appeared to have a positive correlation and higher densitometry measurements (0.51). The reverse appears to be the same for glutamine residues (-0.51). These data suggests that available loop regions with more prolines or less glutamines could have some affect on antibody recognition. Further study comparing the composition for the entire loop would be needed to determine if a causal relationship exists between the type of residue and the propensity it will be recognized by IgE antibodies.

Arachis species that contain Ara h 2 orthologs with significant changes in the IgE-binding epitopes could be targets for crop improvement or the development of immunotherapy. Variations among the Ara h 2 orthologs were concentrated on the loop connecting α-helices H2 and H3, and could alter the IgE-binding affinity towards epitopes 6 and epitope 7 (Figure 3.4). IgE-binding epitopes were predicted to be present on the surface of Ara h 2 orthologs, including the H2-H3 loop. However, dot immunoblots demonstrated that species outside of section *Arachis* possessed variations in the H2-H3 loop that had substantial reduction in antibody binding (Figure 3.6). Though finding Ara h 2 variants with reduced IgE-binding affinity is one objective towards the goal of the development of a hypoallergenic peanut, further tests would be needed to identify other regions that interact with immune cells, specifically antigen presenting cells and regulatory T-cells (Vickery and Burks, 2009). As shown here, wild species could possess variations to the major allergen Ara h 2 that could be less allergenic. Species with hypoallergenic proteins would be important towards the development of a safer peanut crop.

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CHAPTER 4: General Conclusions

The legume genus *Arachis* contains 80 species including the economically important peanut crop, *A. hypogaea* (Krapovickas and Gregory, 1994; Valls and Simpson, 2005). The monograph for this South American genus taxonomically classified *Arachis* species into nine sections based on data from morphology, crossability, and geographic distribution (Krapovickas and Gregory, 1994). Cytogenetic evidence has indicated that section *Arachis*, the largest section, can be further divided into genome groups, A, B, and D, based on chromosome morphology (Husted, 1933; Smartt et al., 1978; Stalker, 1991). While the majority of species are diploid with a base chromosome number of x=10, tetraploid and aneuploid species have also been identified (Krapovickas and Gregory, 1994; Lavia, 1998; Peñaloza and Valls, 1997). The most well known tetraploid is the peanut crop and wild members of the genus *Arachis* have been considered to be potential genetic resources (Rao et al., 2003). Thus, a clear depiction of species relationships would benefit efforts to improve the crop, which has a narrow genetic background. The monograph depicted an intuitive illustration of the evolutionary and phylogenetic relationships among the *Arachis* sections (Krapovickas and Gregory, 1994). The species relationships proposed by Krapovickas and Gregory (1994) were used as a hypothesis. The relationships described in the monograph were compared to those reflected in the trees that resulted from phylogenetic analyses of partitioned and combined plastid and nuclear genomic regions.

The phylogenies presented in the second chapter partially agree with Krapovickas and Gregory (1994) in regards to the validity of sections *Caulorrhizae*, *Extranervosae*, and *Triseminatae*. The phylogenetic trees based on ITS and combined *trn*T-*trn*F data also demonstrated that the most basal section of genus *Arachis* is section *Extranervosae*. It is followed by the monotypic section *Triseminatae* and section *Caulorrhizae*. The species relationships in and among sections *Arachis*, *Erectoides*, *Heteranthae*, *Procumbentes*,

Rhizomatosae and *Trierectoides* need further investigation, as species from these sections did not resolve as monophyletic clades. Sections *Erectoides, Heteranthae, Procumbentes,* and *Trierectoides* formed a heterogeneous lineage and sublineages did not group according to their taxonomic classification. Sections *Erectoides, Procumbentes, Rhizomatosae, and Trierectoides* were previously recognized as a single unit (Gregory and Gregory, 1979). Among species in section *Arachis*, this study showed it as a lineage that divided into two clades, groups arachis II (A genome clade) and arachis I (non-A genome clade).

Further study is needed to resolve the polytomies found within the terminal lineages to gain a full understanding of relationships within group arachis I and arachis II. DNA sequence information from genomic regions that evolve at high rates from both nuclear and chloroplast genomic regions would be beneficial. Examples of potential chloroplast markers that could be utilized include the *trn*S-*trn*G spacer and the rpl16 intron (Shaw et al 2003), while nuclear regions utilized should be low-copy nuclear genes (Hughes et al., 2006). As a whole, phylogenies are useful in providing information on the diversity within the genus and the relationships among species. The molecular phylogenies produced in the second chapter are among the first generated for the genus and provides a clearer illustration of the relationships among sections and genomes. Thus, the phylogenies from Chapter Two can be utilized for conservation of germplasm and genetic resources for breeding to improve the crop (Koppolu et al., 2010).

The use of wild relatives as a source of genetic material has normally focused on the introgression of genes that can be utilized to resist abiotic stress and diseases (Burow et al., 2001; Foncéka et al., 2009; Stalker, 1980). These types of improvements can benefit farmers in terms

of yield and crop quality. However the benefits of crop improvement should not be limited to producers; it can benefit end consumers as well (e.g. Mou, 2005; Oritz et al., 2007).

The third chapter of this dissertation examines wild species of genus *Arachis* for variations among orthologs of the major peanut allergen Ara h 2. Peanut allergies are a serious health issue, as they can cause severe reactions, such as anaphylaxis. These allergies affect 1.2% of the US population, with the number of children diagnosed with this food allergy on the rise (Sicherer et al., 2003). Ten allergy-causing agents have been identified in the peanut seed, with Ara h 2 being one of two major allergens that are recognized in the majority of peanut-sensitive persons. Most of the studies on Ara h 2 have focused on the crop and its two putative progenitors, *A. duranensis* and *A. ipaensis* (Koppelman et al., 2001; Ramos et al., 2006; Ramos et al., 2008). The third chapter examined variations in Ara h 2 orthologs from 12 wild species across the genus. The size of the Ara h 2 orthologs tended to be larger within section *Arachis,* than those from other sections. The majority of mutations were identified in the H2-H3 loop region, which also contains the immunodominant epitopes #6 and #7 (Stanley et al., 1997). The two epitopes share a common hexapeptide motif, DPYSPS. The presence of the two or more DPYSPS motifs within the H2-H3 loop was more prevalent in species from section *Arachis*. Orthologs from the species that were resolved in the group erectoides lineages in the second chapter contained hexapeptide motifs that contained an S to G variation (DPYGPS) or were truncated (DPY). Species from two of the three lineages identified at the base of the *Arachis* phylogeny, *A. macedoi* and *A. triseminata*, possessed orthologs with loops that lacked a complete hexapeptide motif. While linear and conformational epitope programs consistently predicted the H2-H3 loop to contain IgE-binding epitopes, dot immunoblots showed reduced antibody binding to *A. guarantica* and *A. macedoi* peptides when compared to the peptide from *A. hypogaea.*

Thus, wild species of genus *Arachis* are not only genetic resources that can be used to improve the crop by providing genes for resistance to disease and abiotic stress (Rao et al., 2003); they could also be used to improve the crop to benefit the consumer.

Further study of the proteins would be needed to understand the allergy-causing potential, or lack thereof, for Ara h 2 orthologs from wild species. The dot immunoblot assay examined differential antibody binding to peptides that represented the first 15-16 residues of the H2-H3 loop. However, the remaining epitopes of Ara h 2 are distributed along the length of the protein. Experimental data for the whole protein would be needed to examine the effects of the variations and to supplement the bioinformatics approaches used here. Ara h 2 protein could be isolated from seed or expressed recombinantly (Koppelman et al., 2001; Lehmann et al., 2003). Both methods were attempted for this dissertation; however, sufficient amounts of purified protein were not obtained. Of the two methods, recombinant protein expression would produce larger amounts of protein. The seeds from wild species of *Arachis* tend to be substantially smaller when compared to the crop, and as a consequence, protein isolation would require more seeds and would not be as efficient.

In addition to the understanding the effect variations to the Ara h 2 orthologs have on their allergenic potential, further study is also needed to understand the allergens interaction with other macromolecules that could lead to the development of a hypoallergenic crop. Currently, there is little information on the factors that influence the immune system to develop IgE antibodies against specific food proteins (Bredehorst and David, 2001). The majority of food proteins recognized as allergens have often been glycoproteins, including Ara h 2 (Burks et al., 1991). Even though the IgE-binding regions of Ara h 2 have been found to reside within the protein portion of this glycoprotein (Stanley et al., 1997), carbohydrates that maybe present with

Ara h 2 may increase its recognition as an allergen. Lipids may also play an important role the identification of Aar h 2, in addition to other plant proteins, as food allergens (Thomas et al., 2005). The variations observed in the Ara h 2 orthologs work in concert with these macromolecules, and could affect the recognition of it as an allergen. The role of carbohydrates' and lipids' influence on the recognition of plant proteins, such as Ara h 2, by the innate immune system and subsequent development of IgE-producing B-cells needs to be further studied. Results from such studies would be valuable not only in the treatment of food allergies in general, but also in its prevention.

Orthologs of Ara h 2 that are found to be hypoallergenic could be utilized for crop improvement (Ramos et al., 2008). Using genes from wild relatives to produce a safer peanut cultivar would be preferred over the use of RNA interference (Dodo et al., 2005; Dodo et al., 2008), which would require the development of a transgenic peanut. Wild species that are identified to have hypoallergenic variations of Ara h 2 could be used in breeding programs, which would be most efficient to species closely related to the crop (Foncéka et al., 2009). Gene introgression of distantly related species would require more time and effort on the part of the breeder (Rommens et al., 2007); however, this method would still be preferred to the development of a transgenic crop. Alternatively, these species could be developed specifically for the treatment of peanut allergies.

Research Influence on my Teaching Philosophy

As a student, I felt that science was often taught as a set of facts. However, science is not a set of facts that have to be learned. It is a process of discovery. During my graduate education, I have gained a greater appreciation for that process and have learned more through

participating in research. I have also had the opportunity to share my enjoyment of science though teaching laboratory classes and mentoring undergraduates in Dr. Hilu's lab.

As a teacher, I would like to foster the same appreciation for science in my students by showing them that it is more than just a set of facts that has to be learned. By allowing them to also learn science through experimentation, I think they will be able to see that it is process of discovery and a dynamic subject. Additionally, I would hope that my students would be able to see that conducting research is not limited to the stereotypical Caucasian male in a lab wearing a white lab coat. I hope that I could lead by example that anyone can be a scientist, regardless of gender, ethnicity, and background. My hope is my students not only appreciate the subject, but would also see it as something they can do as well.

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APPENDIX A: Acknowledgements

I would like to thank my co-authors on *Species, genomes and section relationships in genus Arachis (Fabaceae): A molecular phylogeny* (Chapter 2), which was recently submitted for publication in *Plant Systematics and Evolution*. Drs. H. Tom Stalker and Shyam P. Tallury from North Carolina State University provided DNA and leaf material from *Arachis* species. Dr. Dietmar Quandt from Rheinische Friedrich-Wilhelms-Universität provided assistance and guidance on sequence alignment phylogenetic analyses.

I would like to also thank Dr. Roy Pittman from the USDA Southern Regions PI station for providing DNA and seed material from *Arachis* species, specifically section *Trierectoides* species, Deborah Wiley for growing and maintaining plant samples, Chelsea Black and Daniel Serrano Vople for their assistance in laboratory work.

The work towards *Species, genomes and section relationships in genus Arachis (Fabaceae): A molecular phylogeny* (Chapter 2) was partially funded by the Virginia Academy of Science, the American Society of Plant Taxonomist, and the Virginia Tech Graduate Research Development Program.

I would like to thank William Joseph Allen, graduate student in the Department Biochemistry at Virginia Tech, for Molecular Dynamics work on the Ara h 2 ortholog homology models from wild *Arachis* species.

APPENDIX B: Homology Model Assessment

Table B.1 Summary of Ramachandran plots for Ara h 2 orthologs models assessed by PROCHECK.

Species	Length	Percent of Residues in			
		Favored	Additionally Allowed	Generously Allowed	Disallowed
A. batizocoi	165	84.4	12.2	3.4	0.0
A. dardani	155	86.4	11.6	1.4	0.7
A. duranensis/ Ara h 2.01	160	88.1	9.8	2.1	0.0
A. glandulifera	170	86.0	12.0	1.3	0.7
A. guarantica	160	84.5	12.7	2.8	0.0
A. ipaensis/ Ara h 2.02	172	85.4	10.6	3.3	0.7
A. macedoi	156	84.6	11.9	2.8	0.7
A. palustris	176	79.9	16.9	3.2	0.0
A. paraguariensis	155	87.7	11.6	0.7	0.0
A. pintoi	158	85.8	11.3	2.1	0.7
A. rigonii	155	86.3	10.8	2.9	0.0
A. triseminata	158	84.7	12.5	0.7	2.1

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Table B.2 Overall Model Quality z-score for Ara h 2 ortholog models assessed by ProSA, with scores from species within section *Arachis* on the left and those from other sections of *Arachis* on the right. For Ara h 2 orthologs from species outside section *Arachis*, the section is noted within parentheses

Species	z-score	Species	z-score
Ara h 6 (1W2Q)	-5.84		
A. batizocoi (*Arachis*)	-3.41	*A. pintoi* (*Caulorrhizae*)	-3.63
A. duranensis/Ara h 2.01 (*Arachis*)	-3.89	*A. paraguariensis* (*Erectoides*)	- 3.92
A. glandulifera (*Arachis*)	-3.53	*A. macedoi* (*Extranervosae*)	-3.15
A. ipaensis/ Ara h 2.02 (*Arachis*)	-3.53	*A. dardani* (*Heteranthae*)	-3.50
A. palustris (*Arachis*)	-3.17	*A. rigonii* (*Procumbentes*)	-3.31
		A. guarantica (*Trierectoides*)	-3.68
		A. triseminata (*Triseminatae*)	-3.21

ANOLEA energy plots for Ara h 6 (PDB ID 1W2Q) and Ara h 2 orthologs

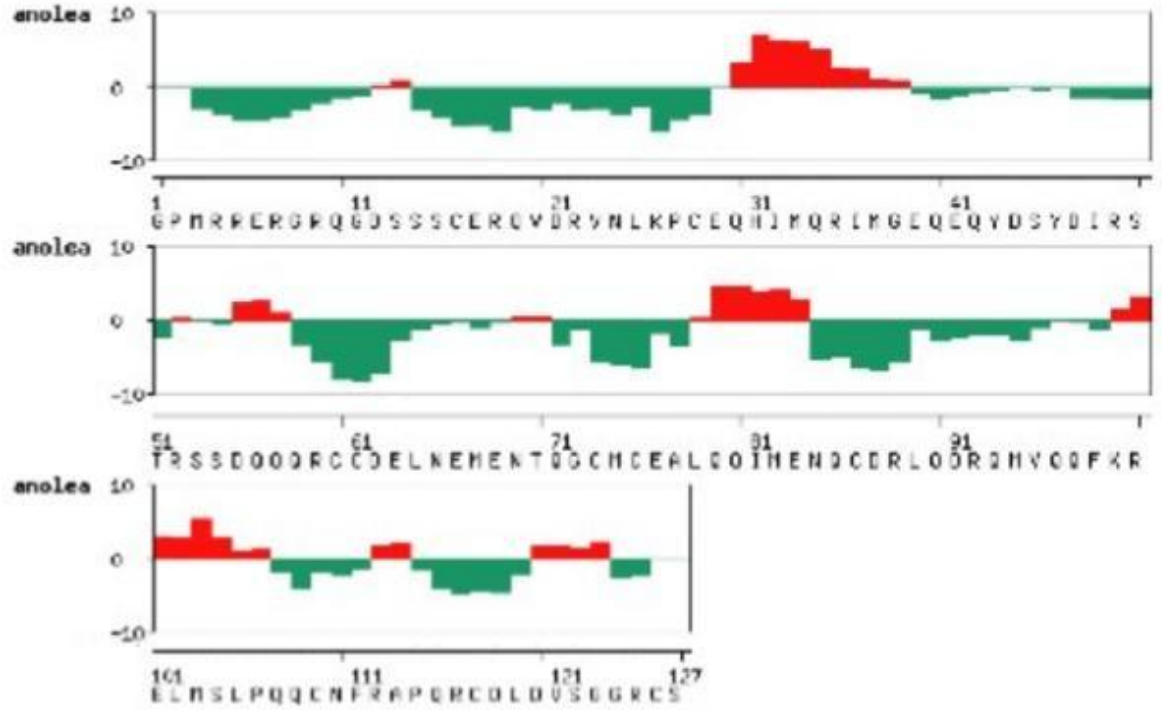


Figure B.1 ANOLEA energy plot for Ara h 6 (PDB ID 1W2Q) template from the crop, *A. hypogaea*. Negative (favorable) energies are in green, while positive (unfavorable) energies are in red.

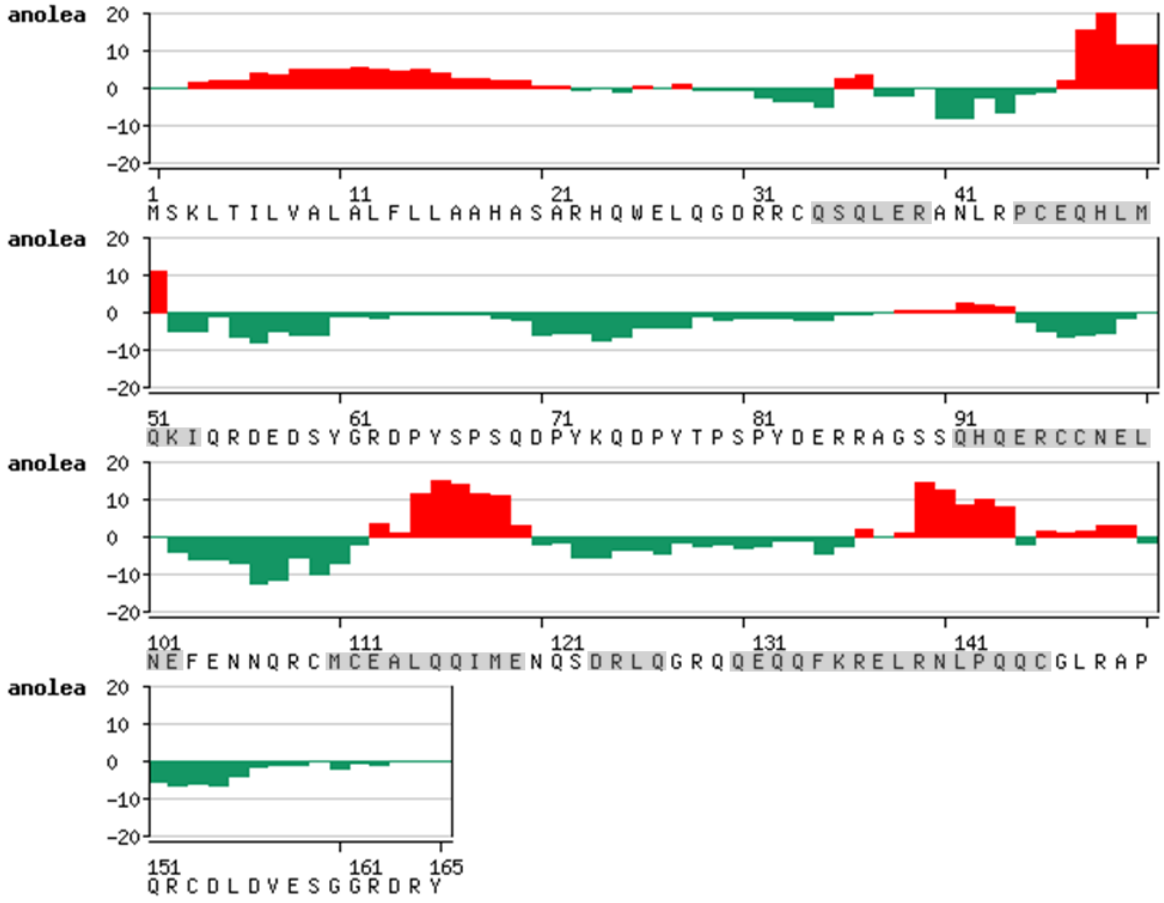


Figure B.2 ANOLEA energy plot for Ara h 2 ortholog from *A. batizocoi* (section *Arachis*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray.

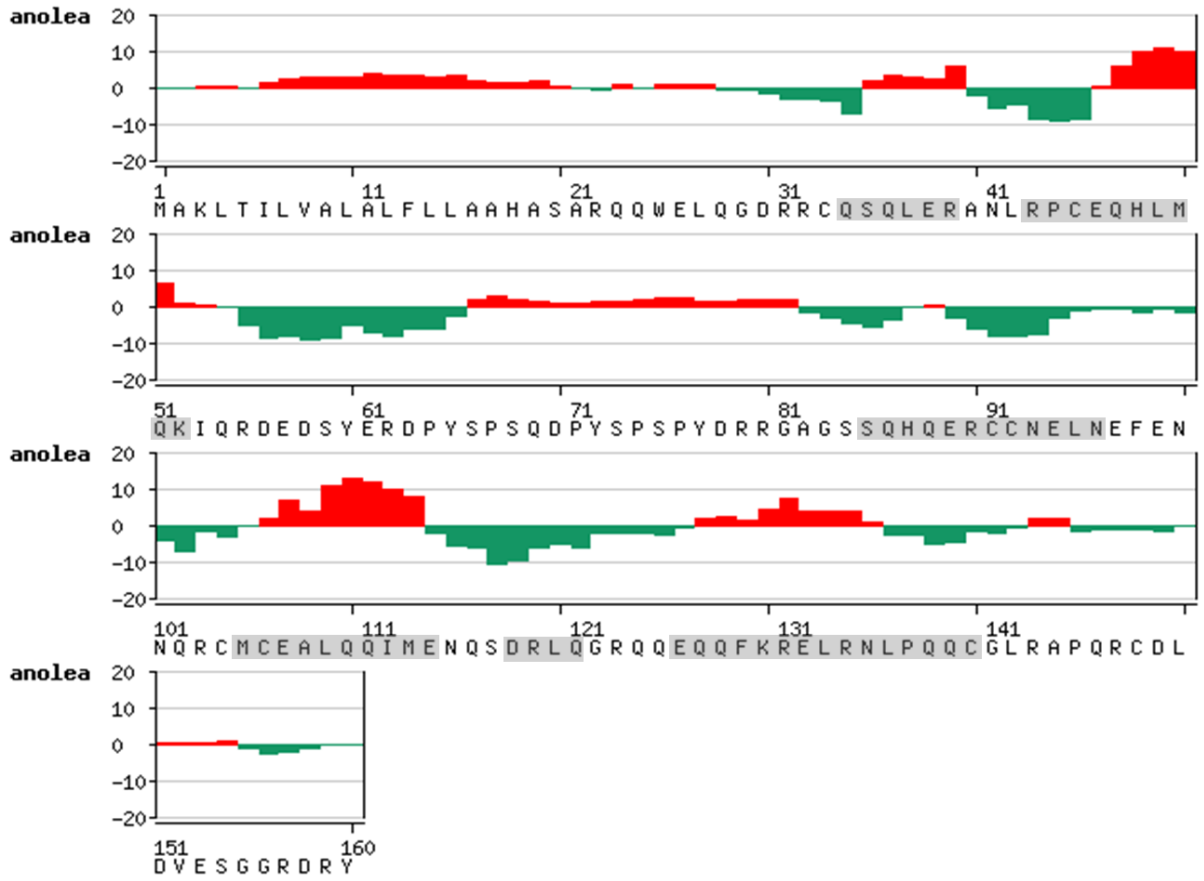


Figure B.3 ANOLEA energy plot for Ara h 2 ortholog from *A. duranensis/A. hypogaea* Ara h 2.01 (section *Arachis*)**.** Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to its extended conformation.

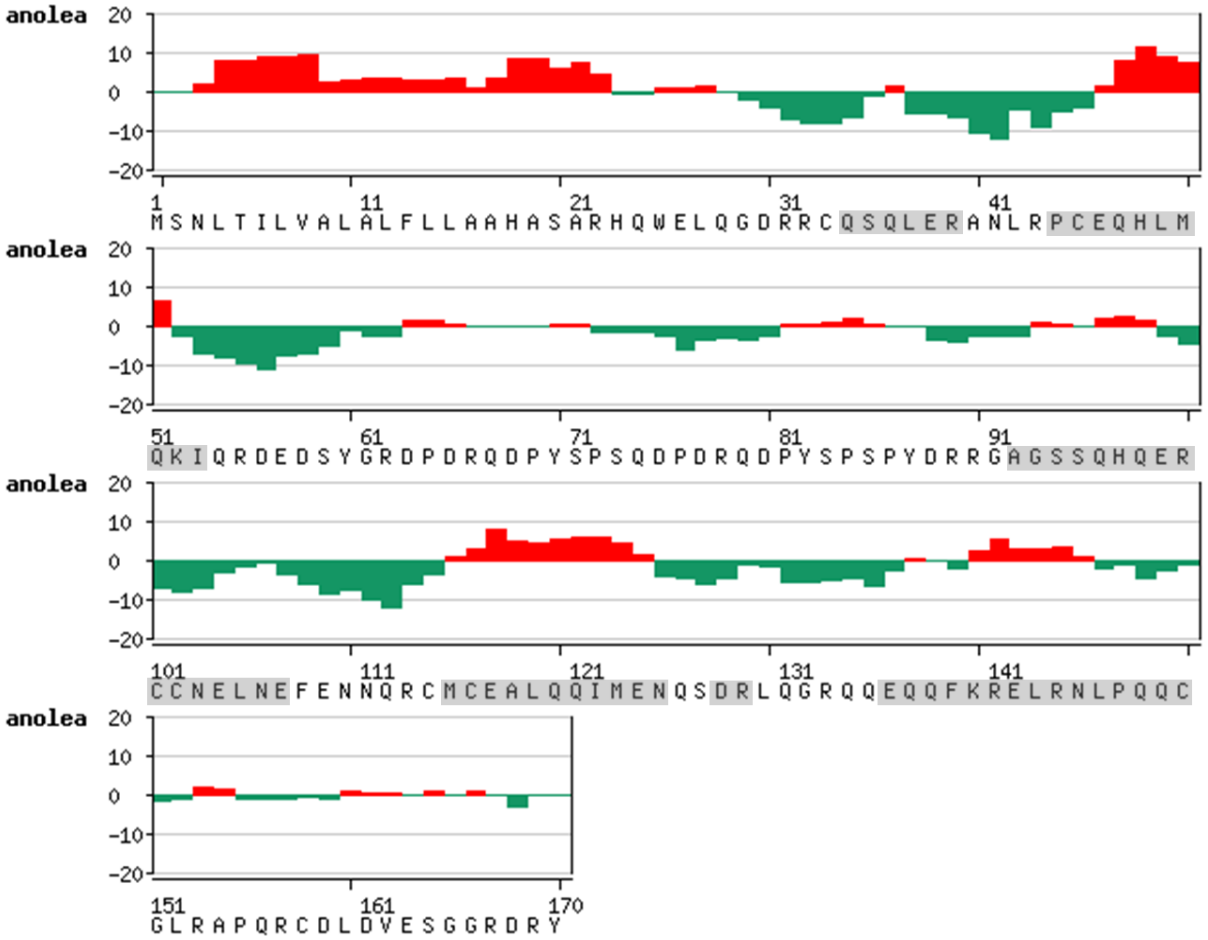


Figure B.4 ANOLEA energy plot for Ara h 2 ortholog from *A. glandulifera* (section *Arachis*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to its extended conformation.

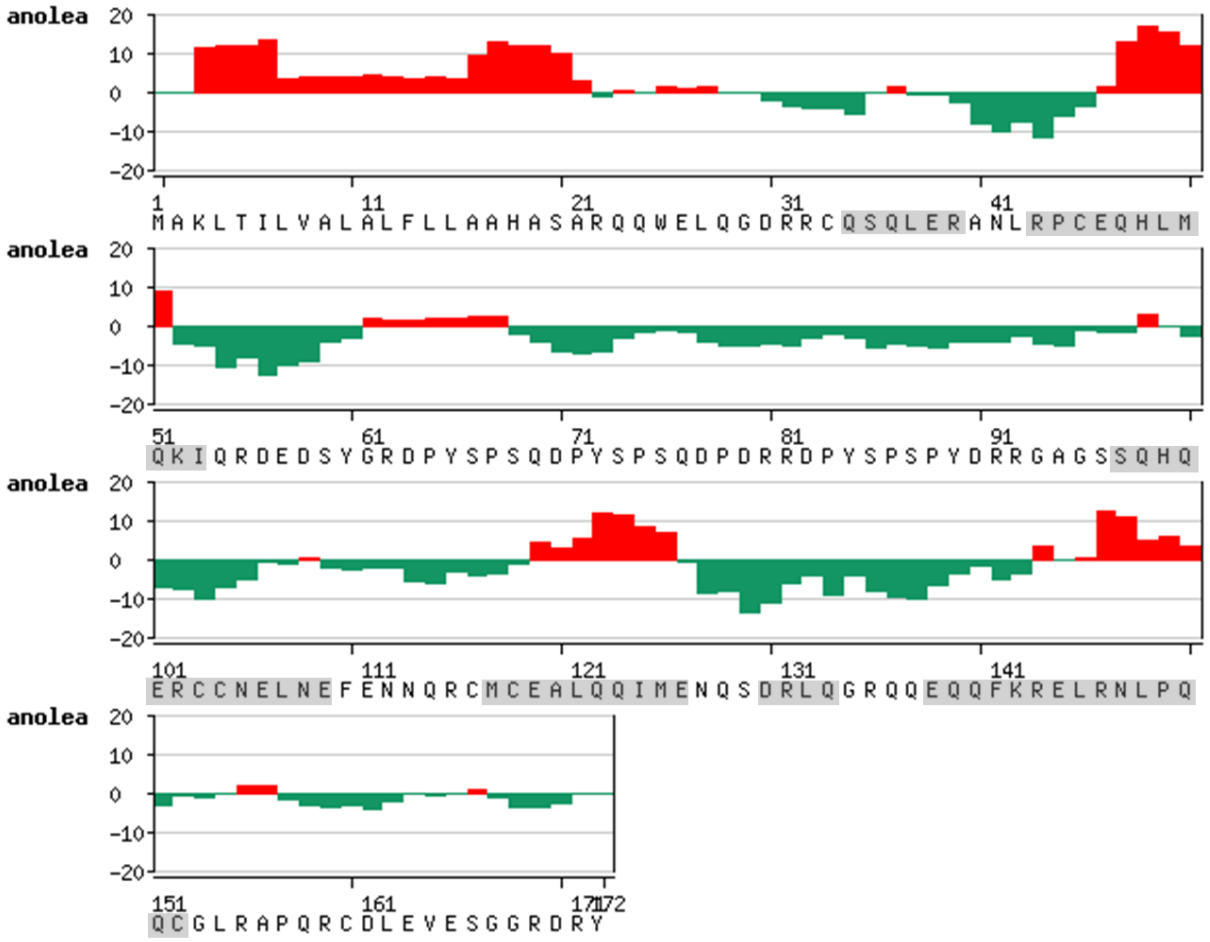


Figure B. 5 ANOLEA energy plot for Ara h 2 ortholog from *A. ipaensis/A. hypogaea* Ara h 2.02 (section *Arachis*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

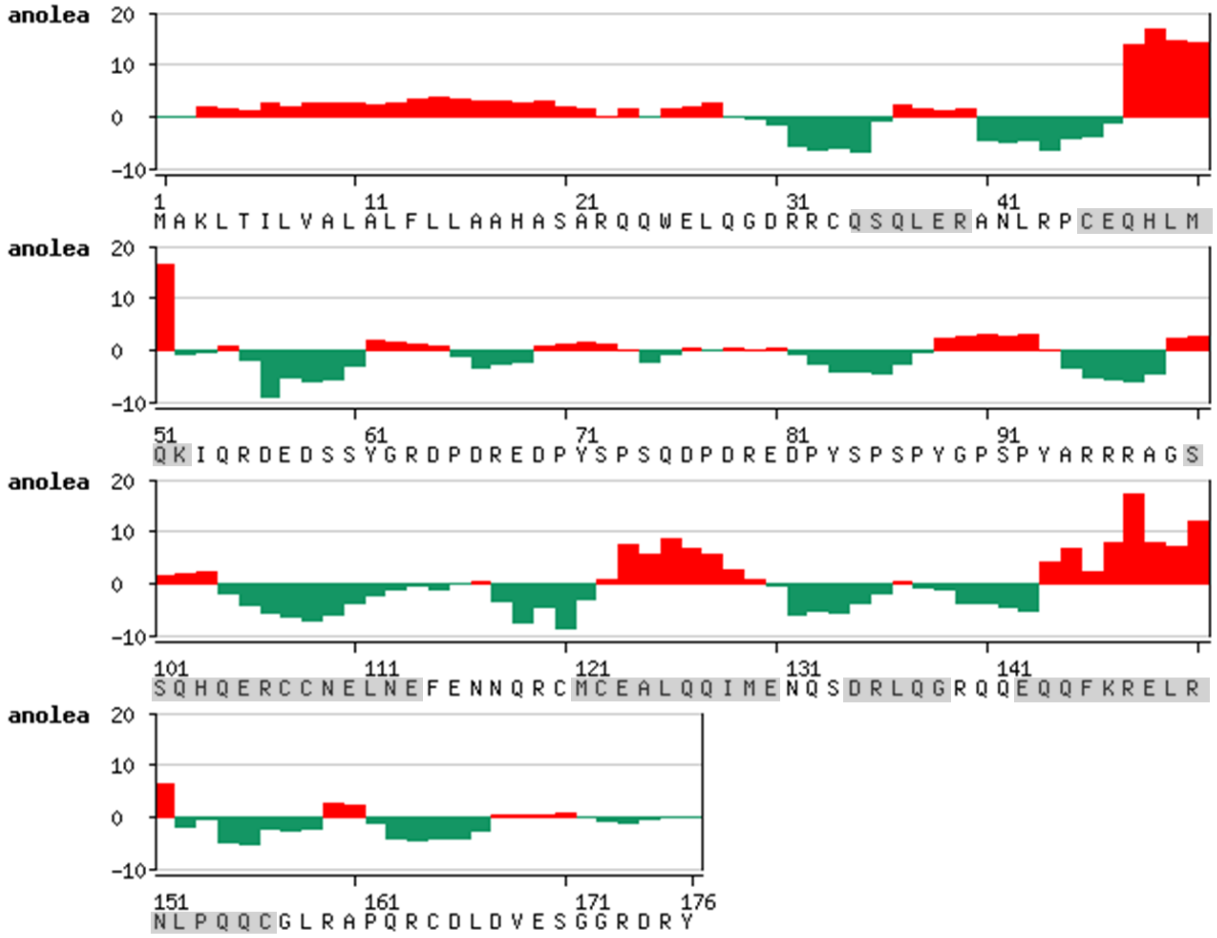


Figure B.6 ANOLEA energy plot for Ara h 2 ortholog from *A. palustris* (section *Arachis*)**.** Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

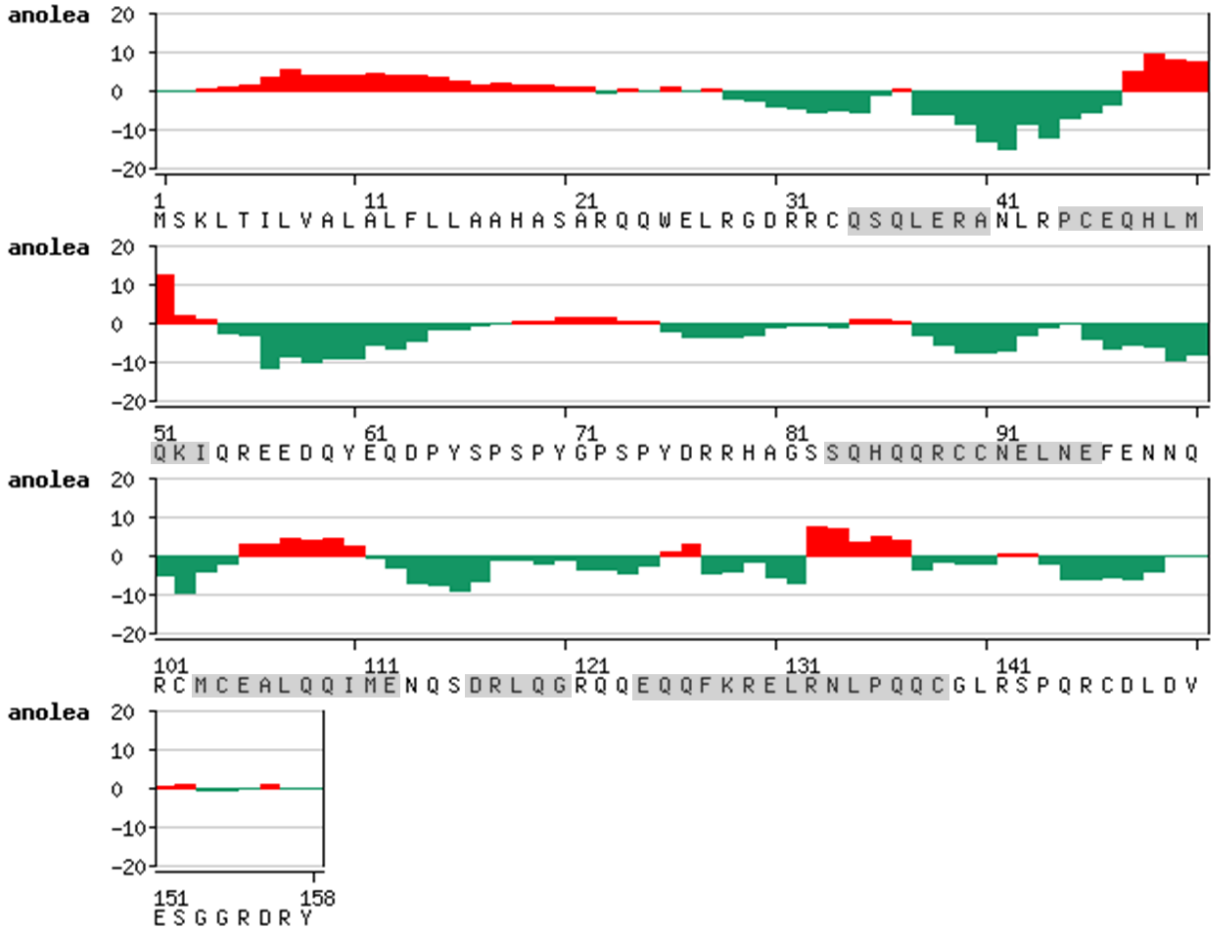


Figure B.7 ANOLEA energy plot for Ara h 2 ortholog from *A. pintoi* (section *Caulorrhizae*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

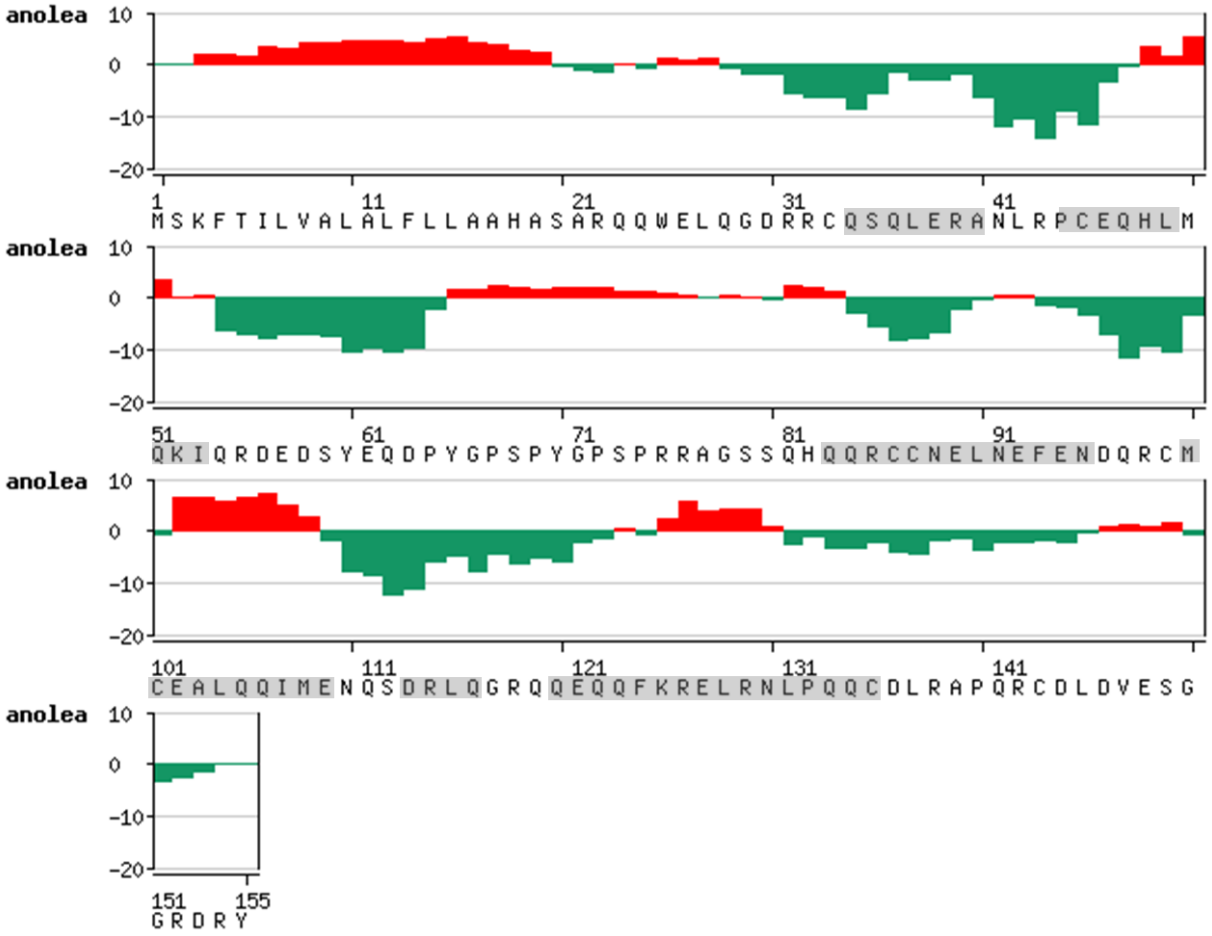


Figure B.8 ANOLEA energy plot for Ara h 2 ortholog from *A. paraguariensis* (sec. *Erectoides*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

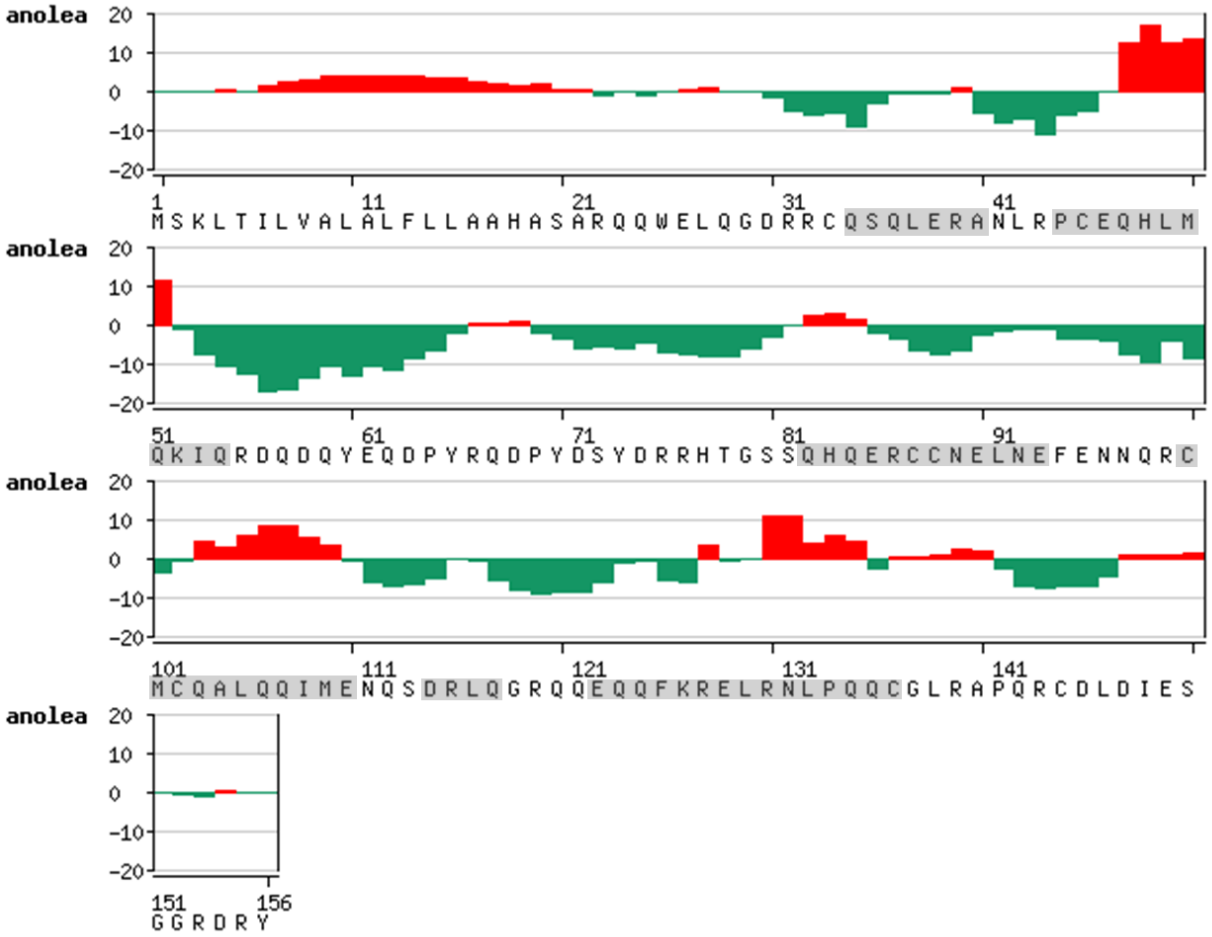


Figure B.9 ANOLEA energy plot for Ara h 2 ortholog from *A. macedoi* (section *Extranervosae*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

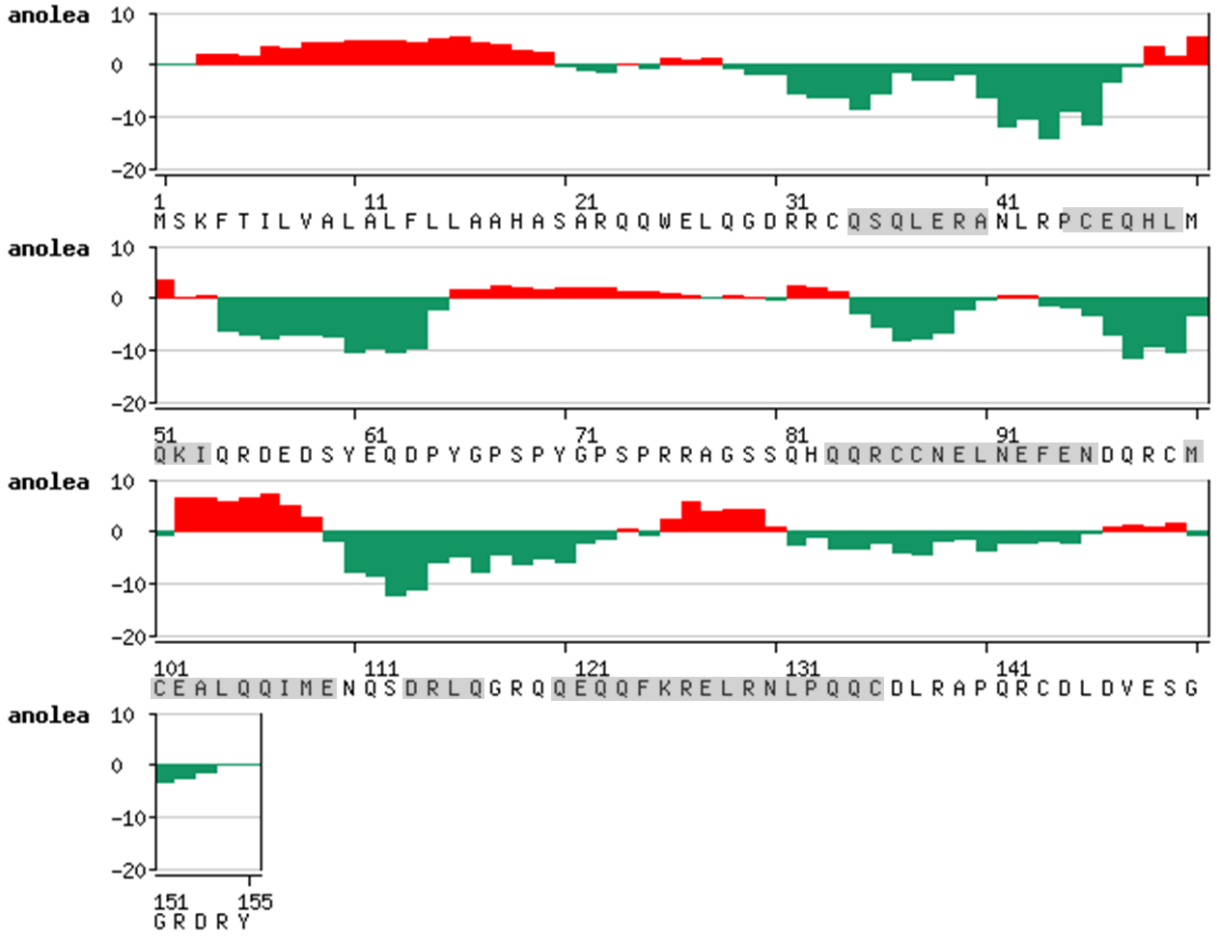


Figure B.10 ANOLEA energy plot for Ara h 2 ortholog from *A. dardani* (sec. *Heteranthae*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

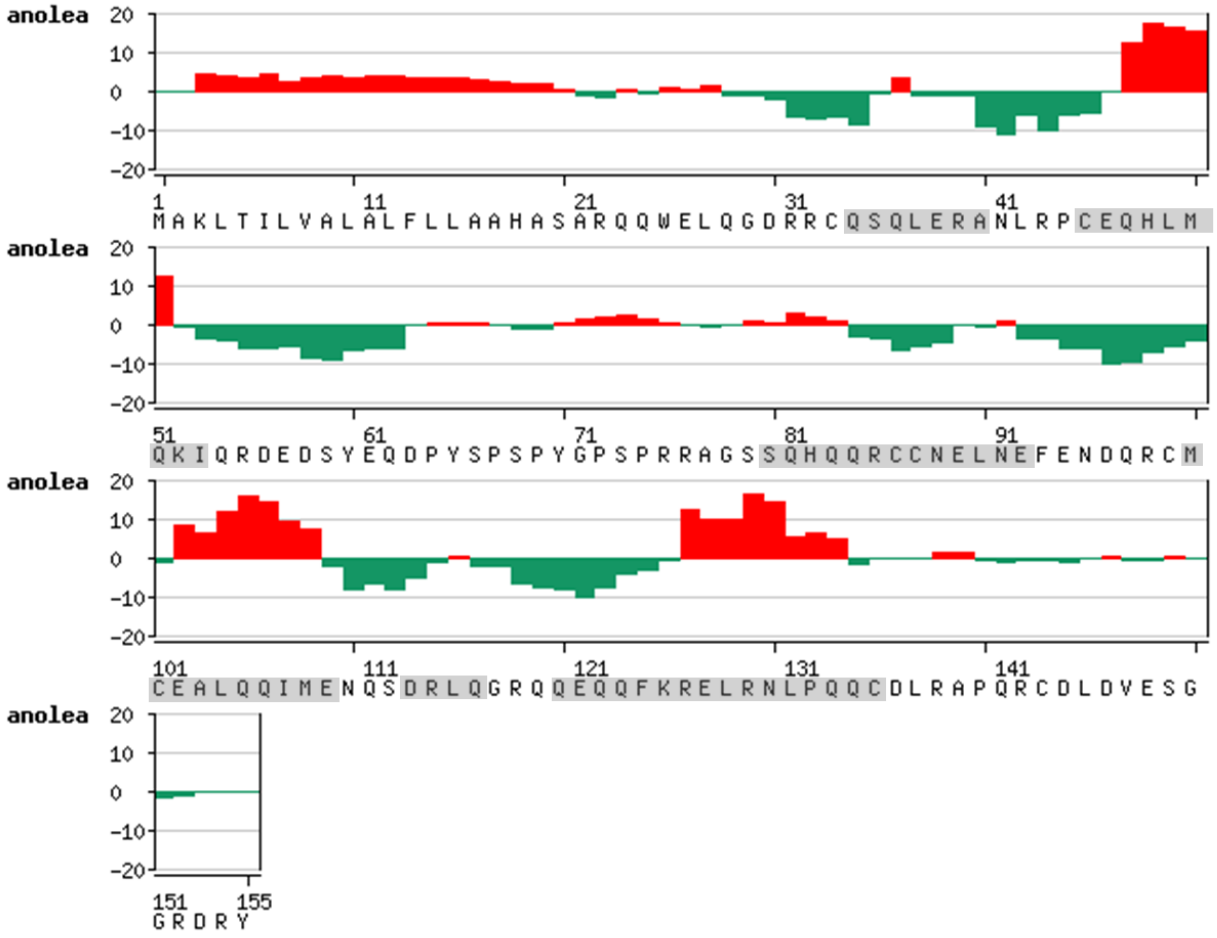


Figure B.11 ANOLEA energy plot for Ara h 2 ortholog from *A. rigonii* (section *Procumbentes*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

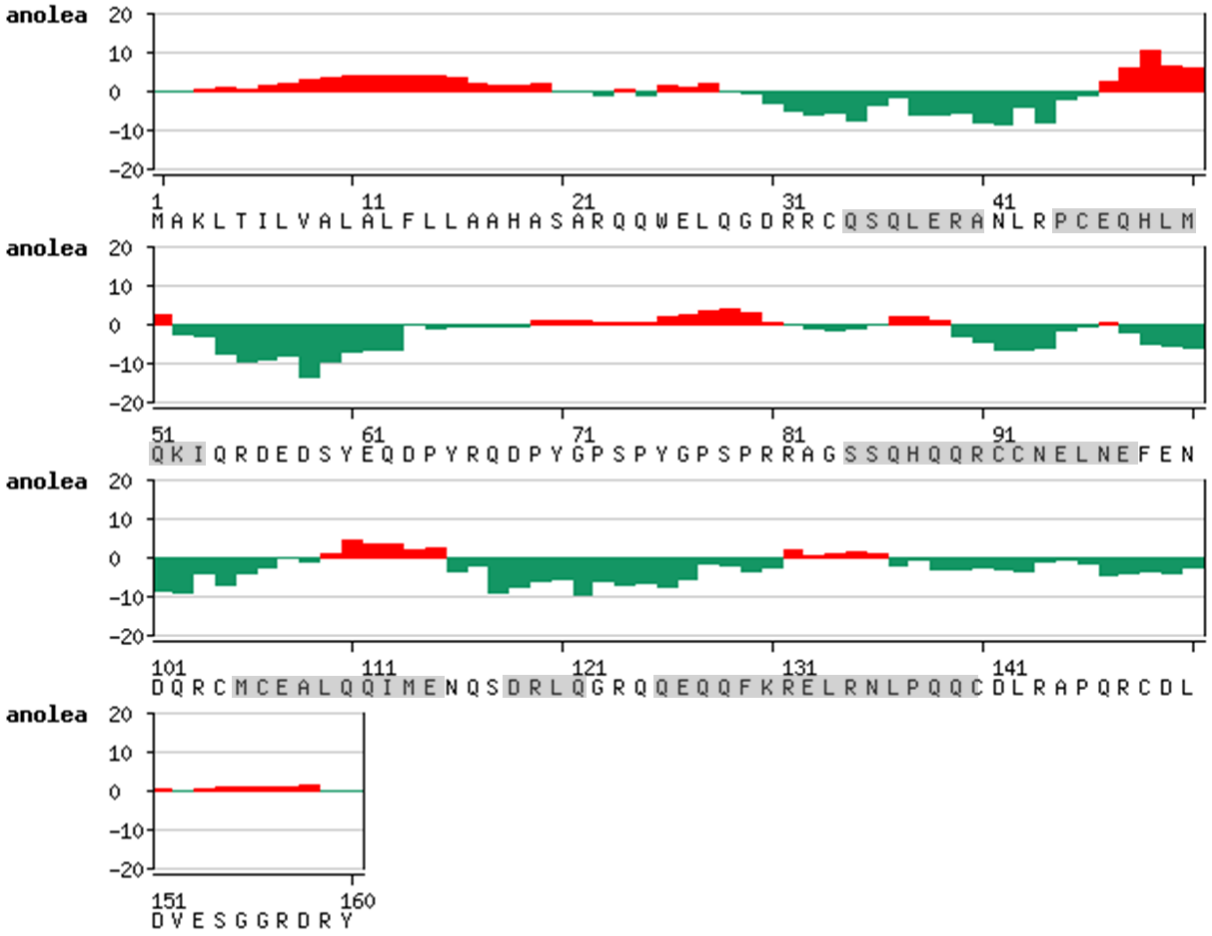


Figure B.12 ANOLEA energy plot for Ara h 2 ortholog from *A. guarantica* (section *Trierectoides*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions.

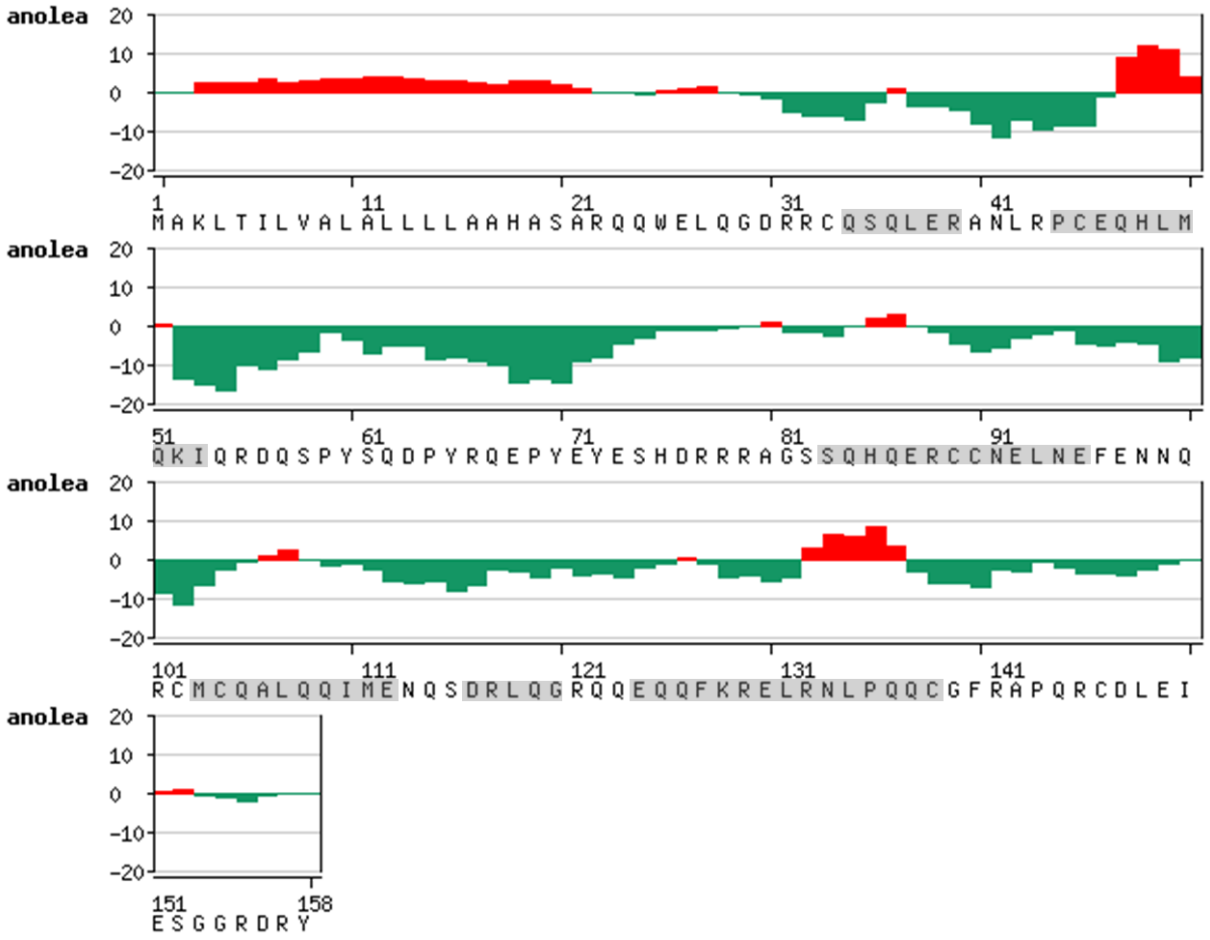


Figure B.13 ANOLEA energy plot for Ara h 2 ortholog from *A. triseminata* (section *Triseminatae*). Negative (favorable) energies are in green, while positive (unfavorable) energies are in red. The α-helical regions are highlighted in gray. For the H2-H3 loop, there were some residues with positive energies that could be related to extended portions**.**

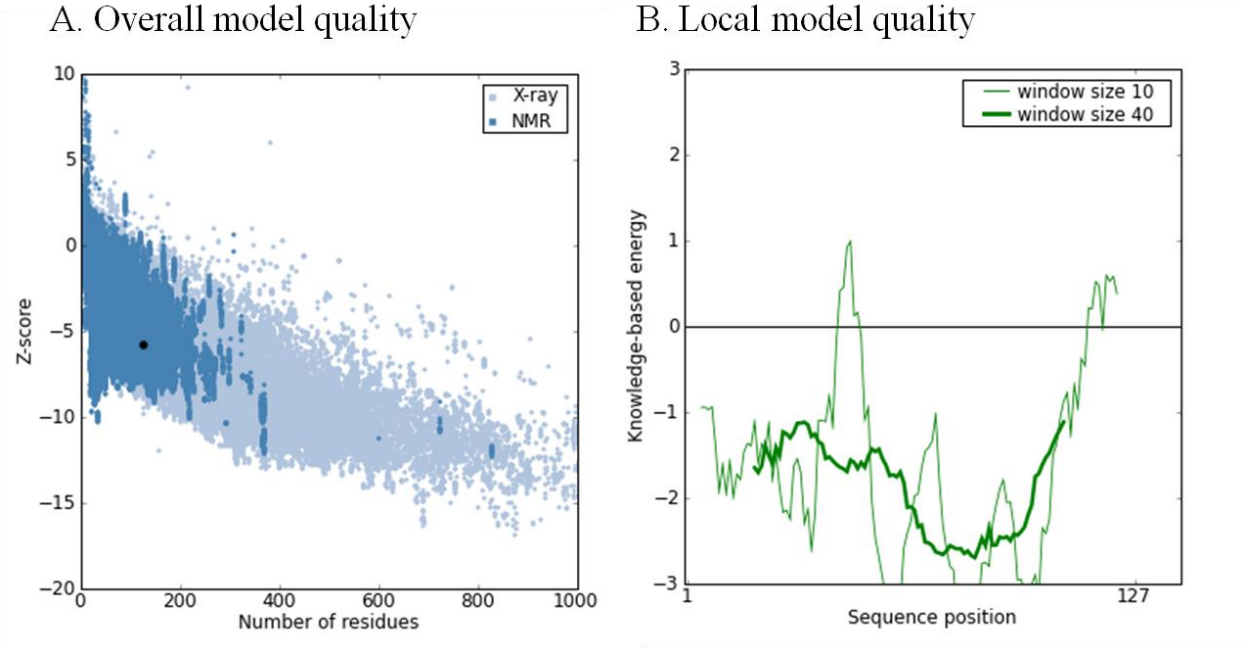


Figure B.14 A. ProSA overall model quality, shows the template Ara h 6 (PDB ID) scored as well as other structures that were determined using NMR. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

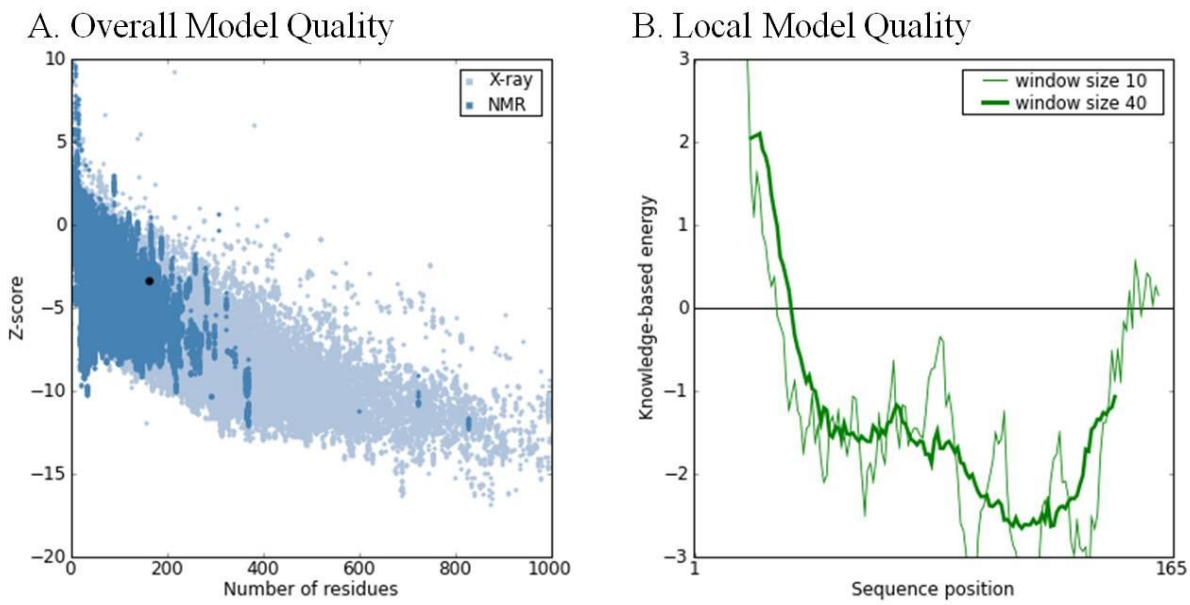


Figure B.15 A. ProSA overall model quality, shows the model of the *A. batizocoi* Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

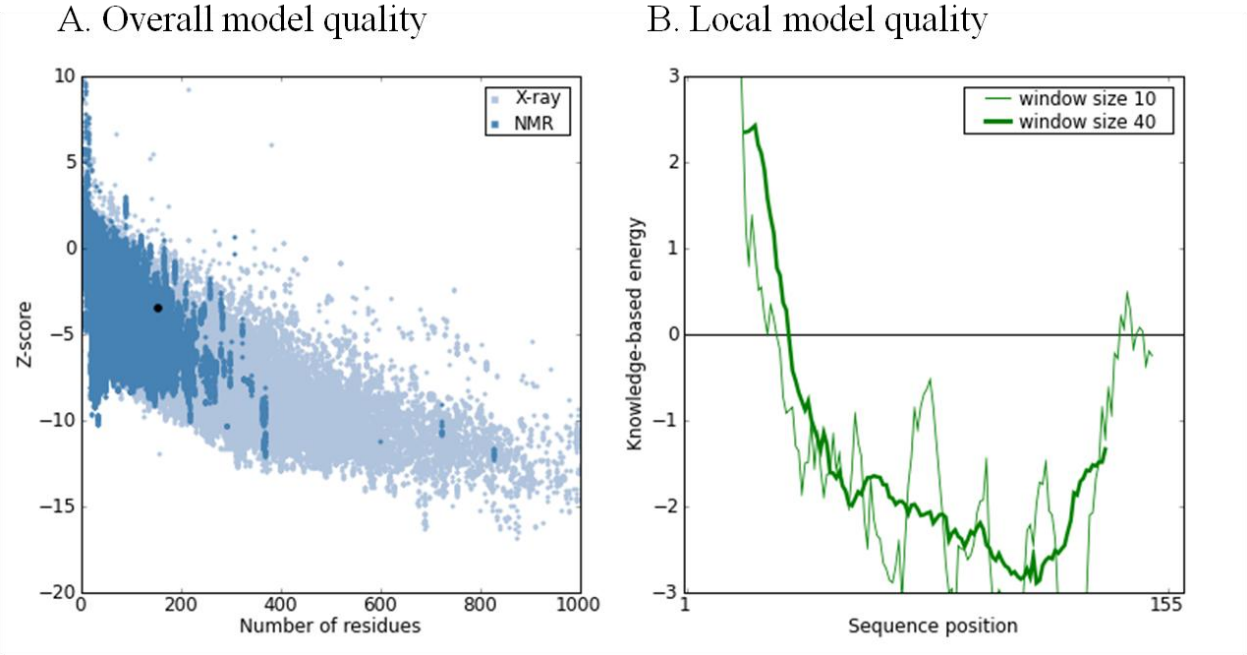


Figure B.16 A. ProSA overall model quality, shows the model of the *A. dardani* (section *Heteranthae*) Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

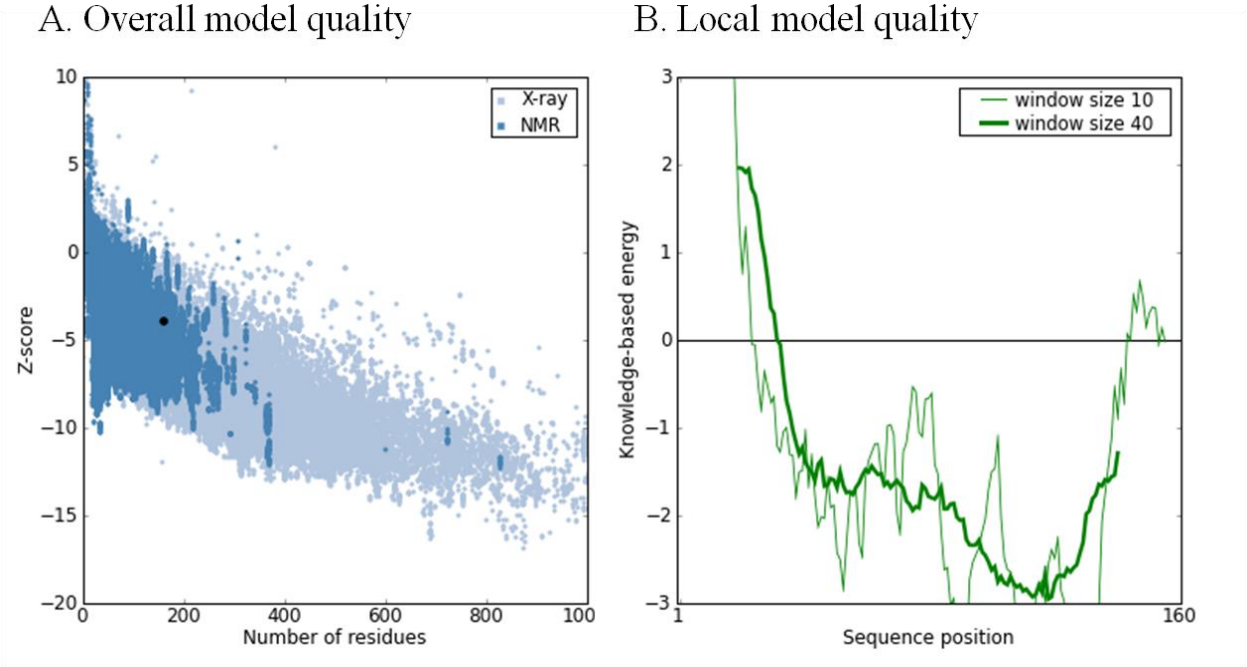


Figure B.17 A. ProSA overall model quality, shows the model of the *A. duranensis*/ Ara h 2.01scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

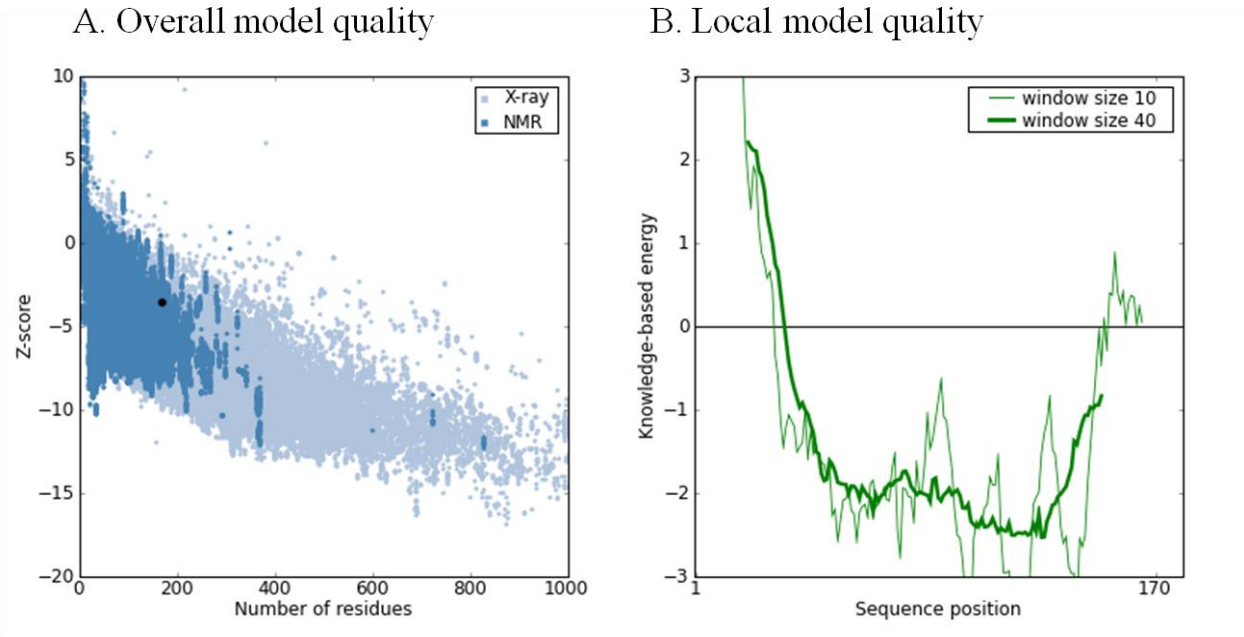


Figure B.18 A. ProSA overall model quality, shows the model of the *A. glandulifera* Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

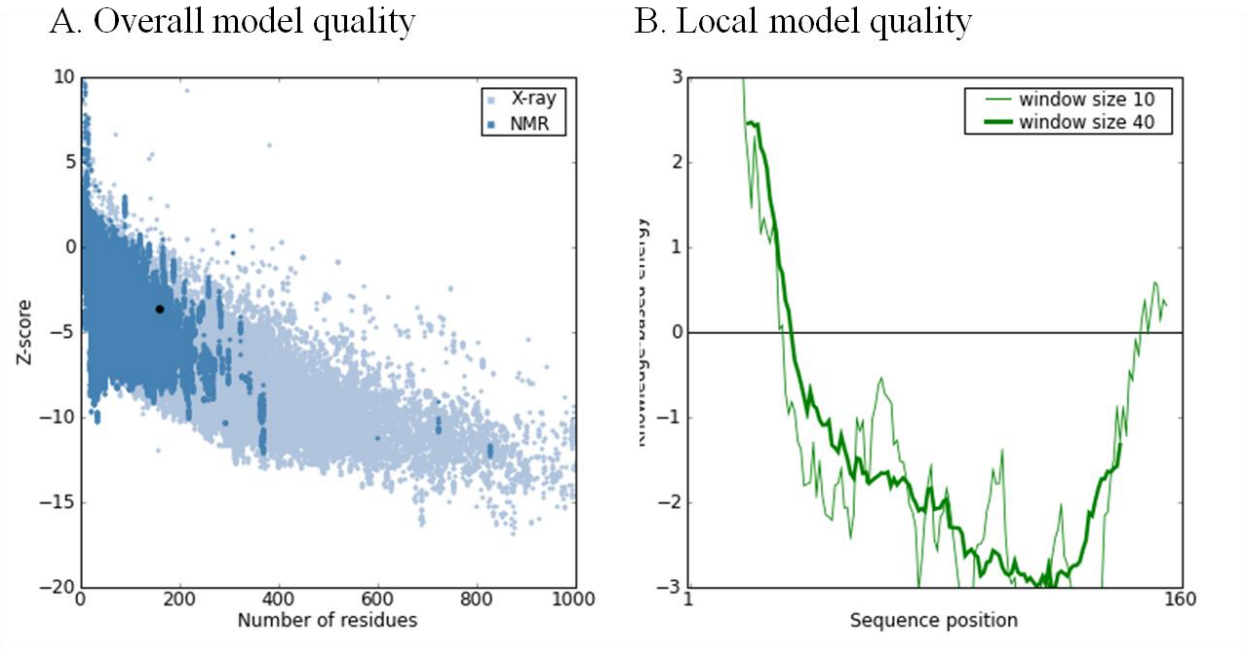


Figure B.19 A. ProSA overall model quality, shows the model of the *A. guarantica* (section *Trierectoides*) Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

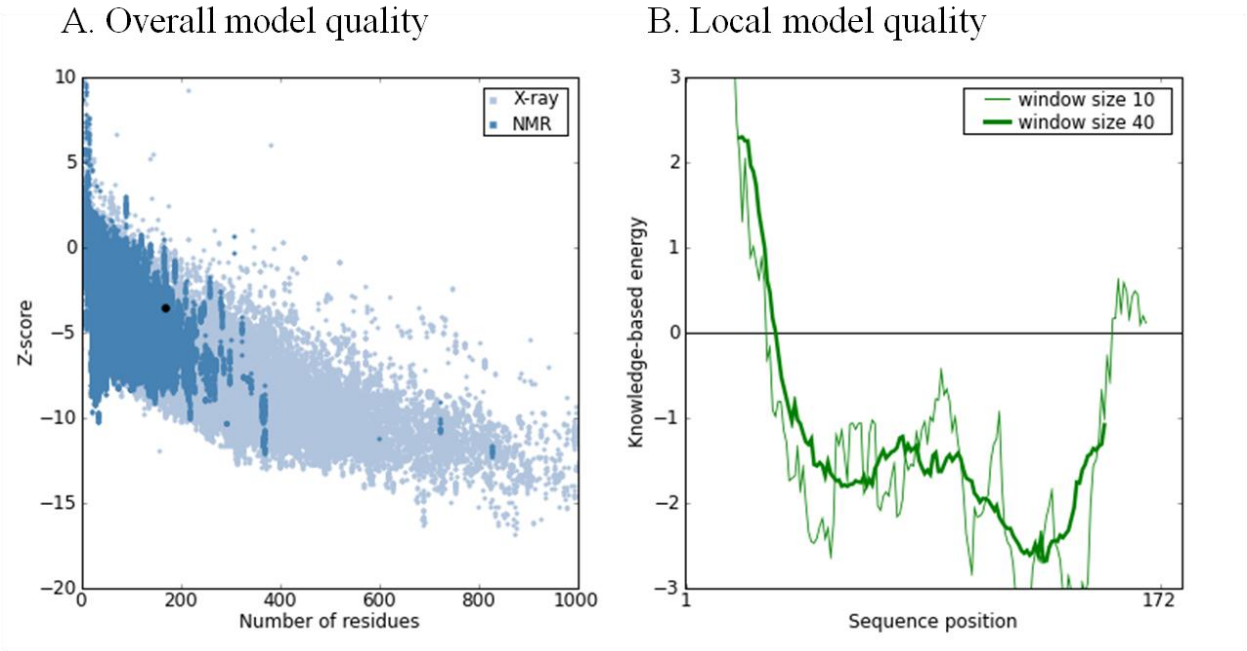


Figure B.20 A. ProSA overall model quality, shows the model of the *A. ipaensis*/Ara h 2.02 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

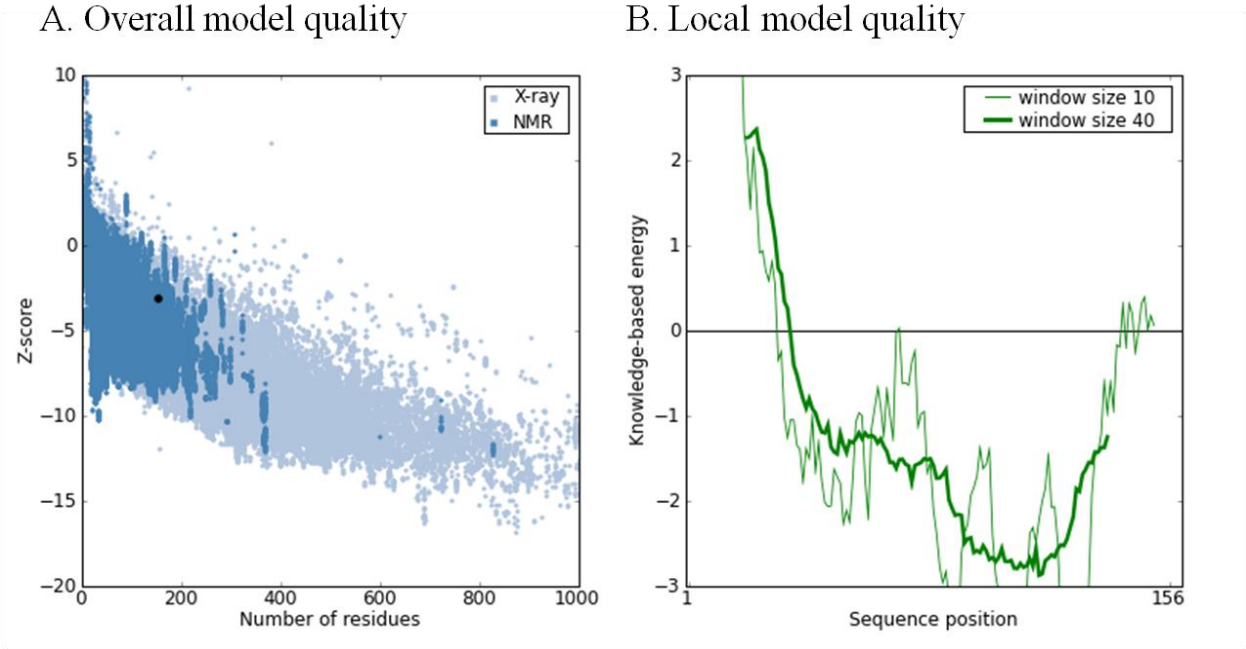


Figure B.21. A. ProSA overall model quality, shows the model of the *A. macedoi* (section *Extranervosae*) Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

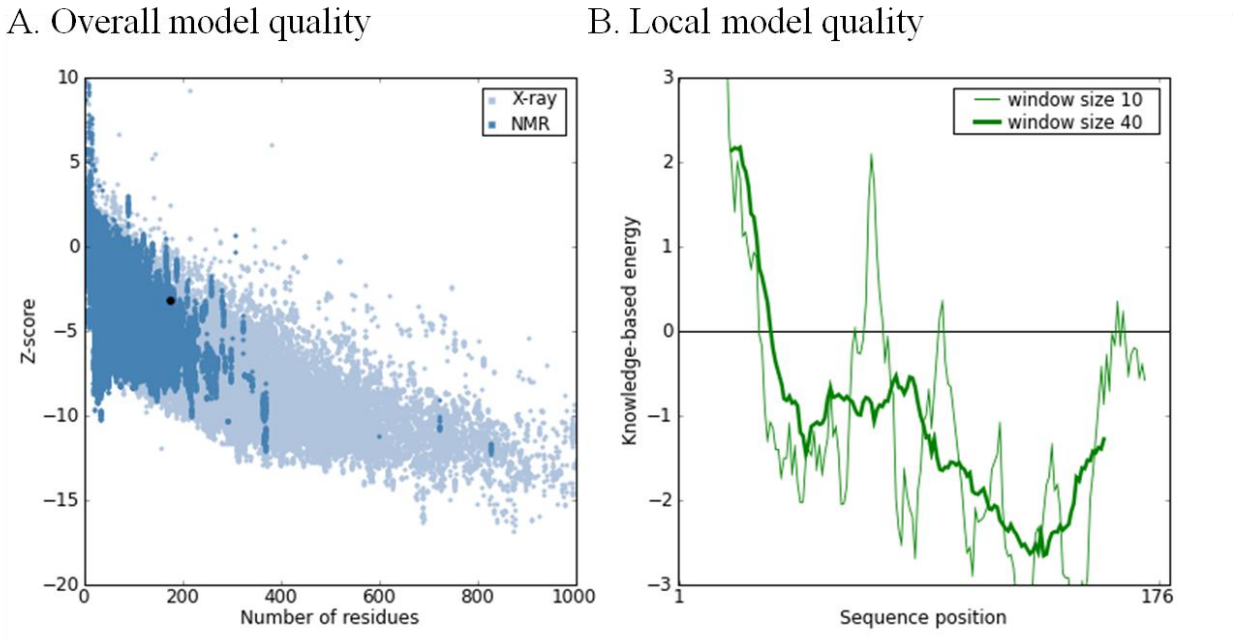


Figure B.22. A. ProSA overall model quality, shows the model of the *A. palustris* Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

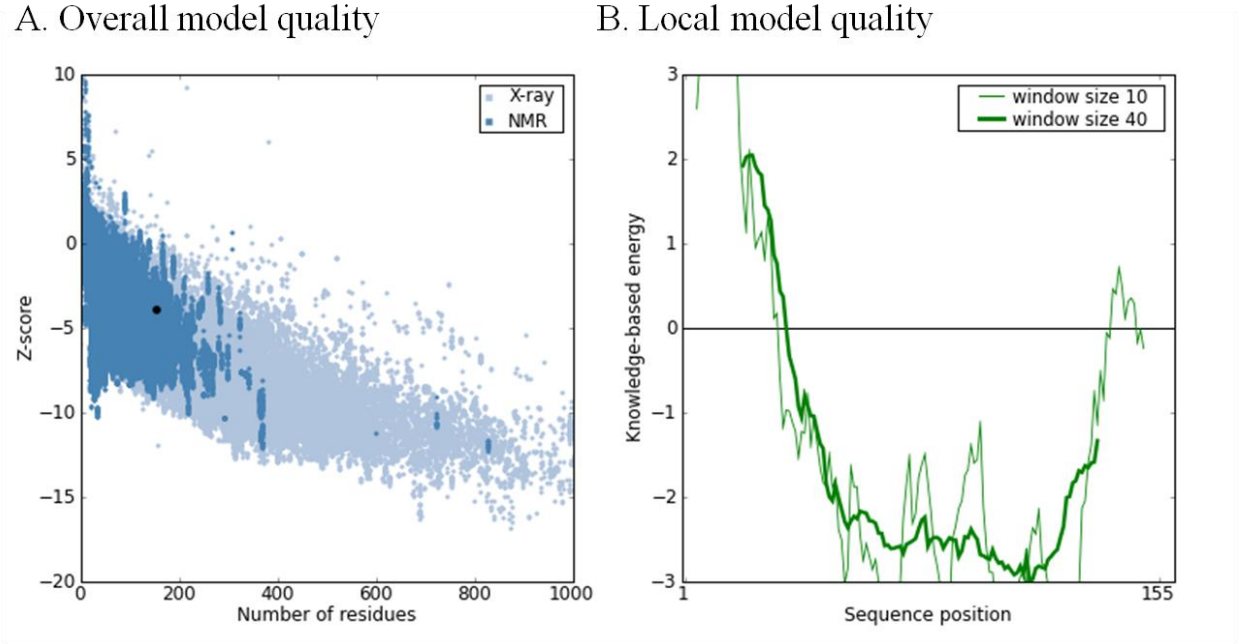


Figure B.23 A. ProSA overall model quality, shows the model of the *A. paraguariensis* (section *Erectoides*) Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini

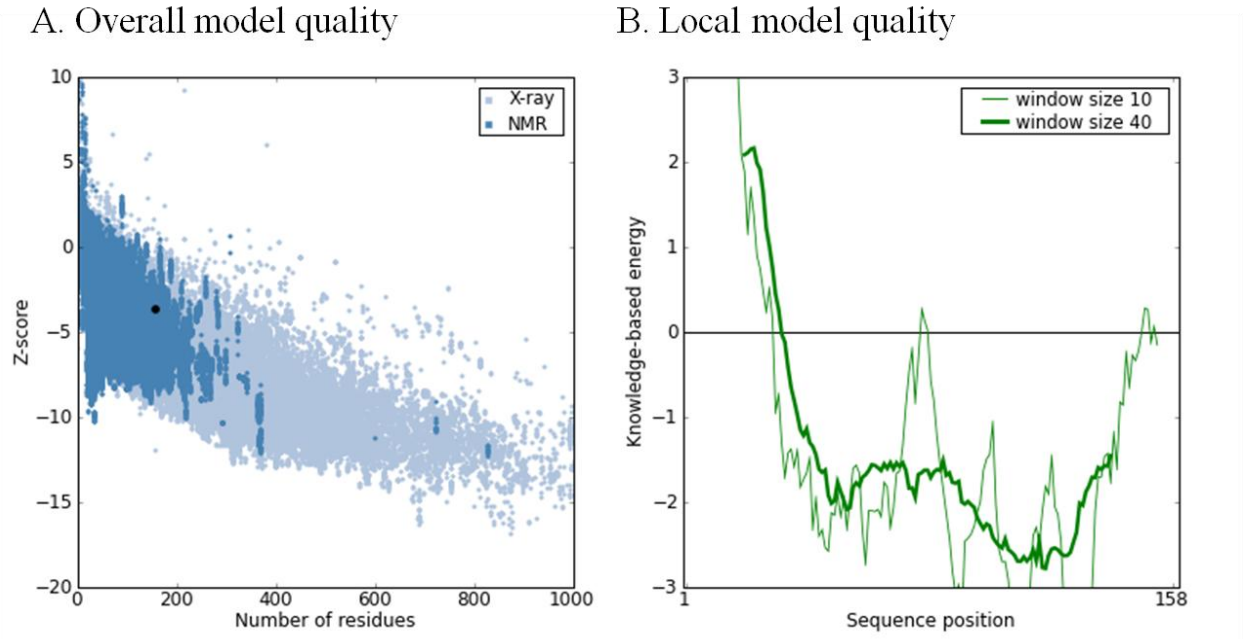


Figure B.24 A. ProSA overall model quality, shows the model of the *A. pintoi* (section *Caulorrhizae*) Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

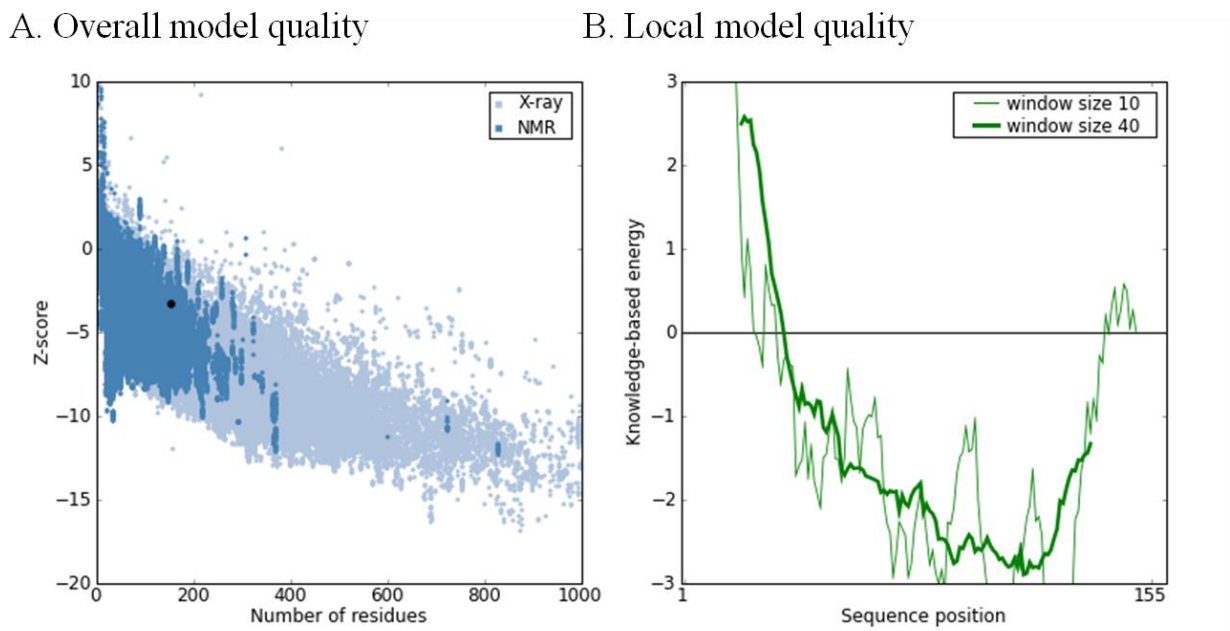


Figure B.25 A. ProSA overall model quality, shows the model of the *A. rigonii* (section *Procumbentes*) Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini

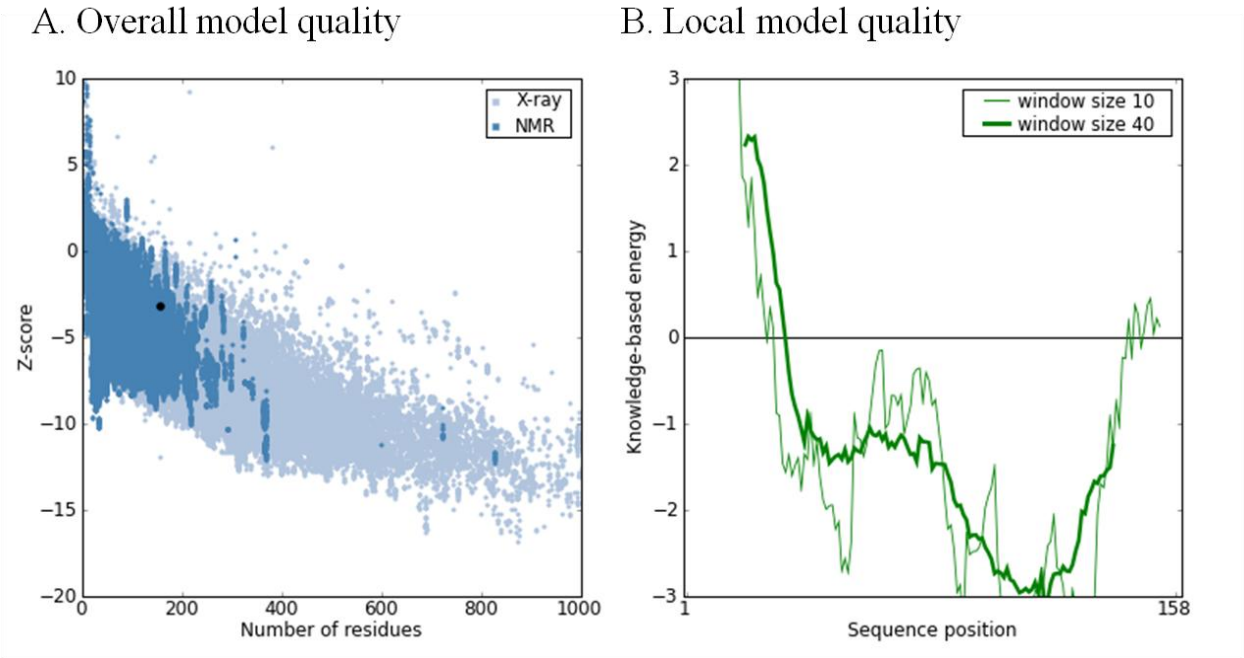


Figure B.26 A. ProSA overall model quality, shows the model of the *A. triseminata* (section *Triseminatae*) Ara h 2 ortholog scored as well as an NMR-determined structure. B. The knowledge base energies for the majority of the protein received favorable (negative) scores for the majority of the protein, with the exception of the N and C termini.

APPENDIX C: Predicted Secondary Structure and Epitopes among Ara h 2 Orthologs from *Arachis* Species

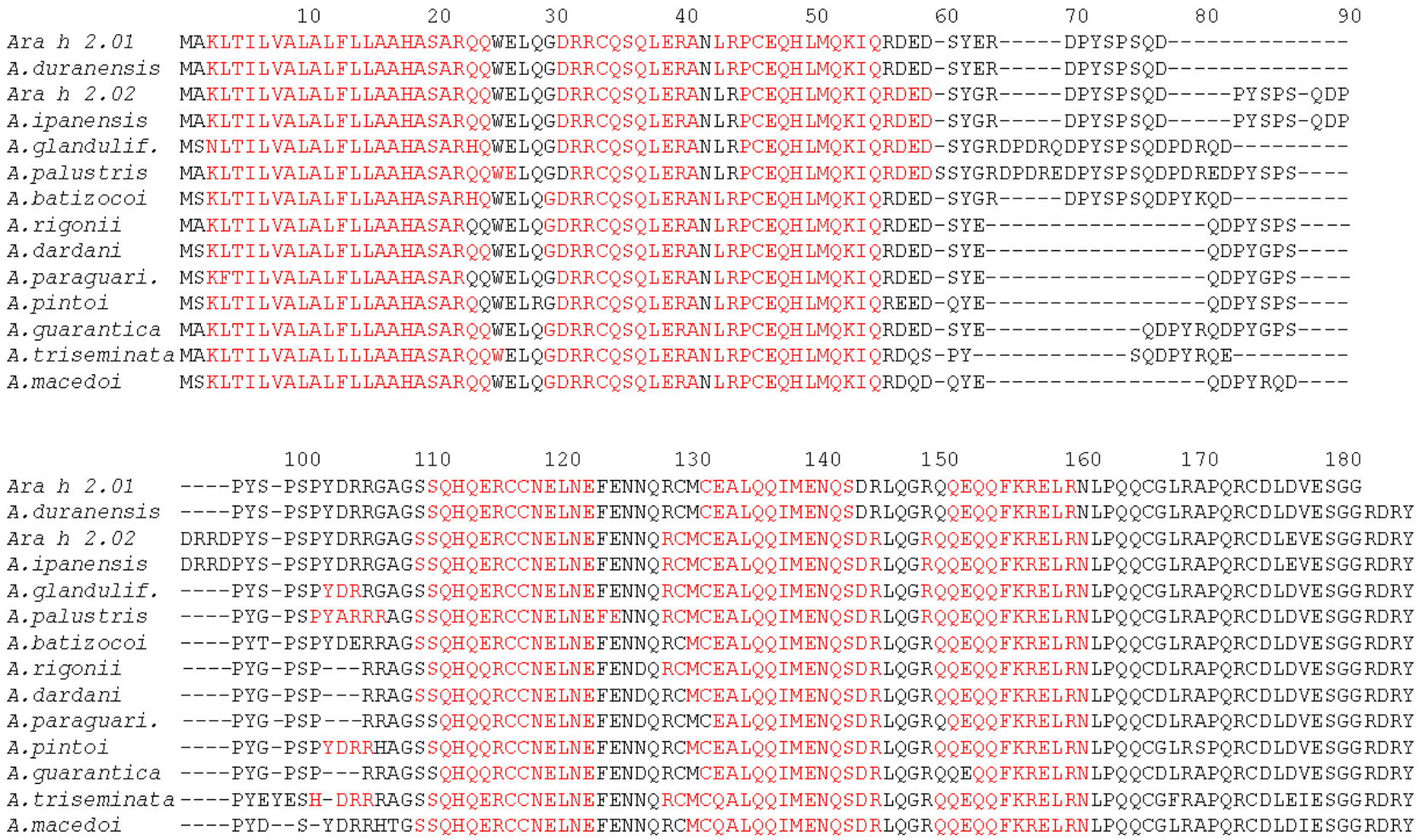
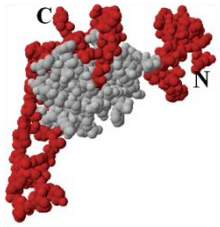
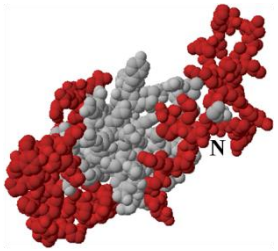
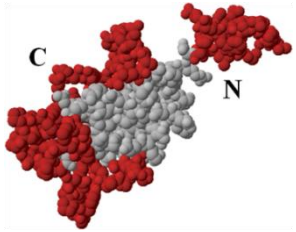
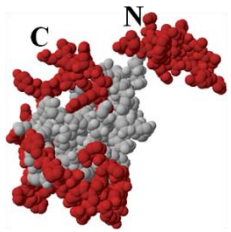
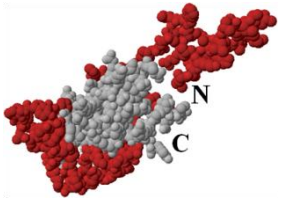
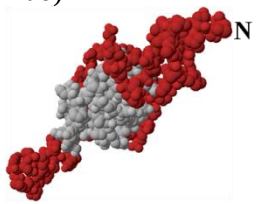


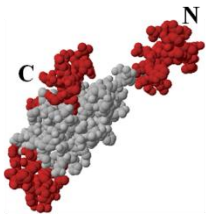
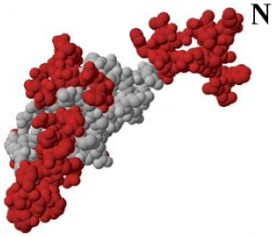
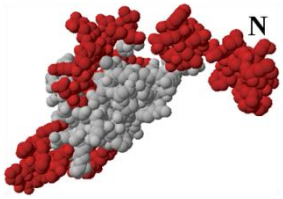
Figure C.1 Secondary structure prediction using PROFsec. Predicted α-helical regions are highlighted in red. Five helices were predicted to be present throughout the protein.

Table C.1 Linear and conformational epitopes predicted by ElliPro server. Residues that were predicted to be potential IgE binding regions are reported for both linear (left) and conformational (right) epitopes.

	Residues predicted as epitopes		Linear Epitopes				
Species	Number	Percent	Positions	Sequence	Score	Conformational	Scores
Ara h 2.01 *(A. duranensis)*	86/48	53.8/28.8	1-29	MAKLTILVALALFLL AAHASARQQWELQG	0.794	S73, P74, S75, P76, Y77	0.975
			64-83	PYSPSQDPYSPSPYDR RGAG	0.77	M1, A2, K3, T5, I6, L7, V8, A9, L10, A11, L12, L14	0.903
			118-128	DRLQGRQQEQQ	0.666	S66, P67, S68, Q69, D70, P71, D78, R79, R80	0.781
			54-59	QRDEDS	0.605	L15, A16, A17, H18, A19, S20, A210.770	0.770
			140-148	CGLRAPQRC	0.603	Q124, E126, Q127	0.712
						G122, R123, Q125	0.702
						Q54, E57, D58	0.642
						R55, D56, E61	0.554
						G81, A82, G83	0.506
A. batizocoi	91/54	55.1/32.7	1-31	MSKLTILVALALFLL AAHASARHQWELQG DR	0.805	A11, L12, F13	0.982
			101-104	NEFE	0.680	T5, I6, L7, V8, A9, L10	0.889
			122-130	DRLQGRQQ	0.647	D83, E84, R86	0.842
			62-75	RDPYSPSQDPYKQD	0.611	L14, L15, A16, A17, H18, A19, S20, A21, R22	0.789
			77-95	YTPSPYDERRAGSSQ HQER	0.604	Q28, G29, D30	0.717
			145-153	CGLRAPQRC	0.589	G127, R128, Q129, Q130	0.689
						A87, G88, S89, Q91, H92	0.623
						R55, D56, D58, S59	0.597

A. glandulifera	95/61	55.9/35.9	1-28	MSNLTILVALALFLL AAHASARHQWELQ	0.814	T5, I6, L7, V8, A9, L10, A11, L12	0.941
			78-97	RQDPYSPSPYDRRGA GSSQH	0.701	S83, P84, S85, P86	0.934
			57-76	EDSYGRDPDRQDPYS PSQDP	0.642	M1, S2, N3, L15, A16, A17, H18, W25, E26, L27	0.791
			153-170	RAPQRCDLDVESGGR DRY	0.598	D161, V162, E163	0.788
			127-135	SDRLQGRQQ	0.592	A19, S20, A21, R22	0.707
						D58, S59, G61, R62, D63, P64, D65, R66, Q67, D68, P69, Y70	0.694
						Q79, D80, P81	0.688
						G91, A92, G93, S94, Q96	0.678
						S71, P72, S73, D75, P76	0.636
						Y87, D88, R90	0.537
						S164, G165, G166, D168	0.512
A. ipaensis	96/68	55.8/39.5	1-29	MAKLTILVALALFLL AAHASARQQWELQG	0.827	M1, A2, K3, L4, T5, I6, L7, V8, A9, L10, A11, L12, F13, L14, L15, A16, A17, H18, A19, S20, A21, R22	0.898
			55-68	RDEDSYGRDPYSPS	0.719	S73, P74, S75, Q76, D77, P78, D79, R80, Y84, D90, R92	0.718
			85-98	SPSPYDRRGAGSSQ	0.660	R91, G93, A94, G95, S96, Q98	0.686
			71-82	PYSPSQDPDRRD	0.629	R55, D56, E57, Y60, G61, R62, D63, P64, Y65, R135, Q137	0.682
			130-137	DRLQGRQQ	0.612	S66, P67, S68	0.618
			154-172	LRAPQRCDLEVESGG RDRY	0.602	Q23, E26, L27, Q28, G29	0.597
						S85, P86, S87, P88	0.576
						L162, E165, S166, G167, G168, R171	0.567

A. palustris	91/50	55.1/28.4	1-31	MAKLTILVALALFLL AAHASARQQWELQG DR	0.812	L4, T5, I6, L7, V8, A9, L10, A11, L12	0.941
			85-99	PSPYGPSPYARRRAG	0.729	G89, P90, S91, P92, Y93, R97	0.882
			54-67	QRDEDSSYGRDPDR	0.651	A16, A17, H18	0.877
			156-164	CGLRAPQRC	0.623	Q24, E26, Q28	0.816
			136-143	LQGRQQEQ	0.574	M1, A2, K3	0.807
			112-119	NEFENNQR	0.550	P85, S86, P87, Y88	0.690
			71-82	YSPSQDPDREDP	0.538	R55, D56, E57, Y61, G62, R63, D64, P65, D66	0.684
						R79, E80, D81, P82	0.609
						A19, S20, A21, R22	0.554
						N109, N112, C156, G157, L158	0.516
A. pintoi (*Caulorrhizae*)	86/33	54.4/20.9	1-29	MSKLTILVALALFLL AAHASARQQWELRG	0.785	M1, S2, K3	0.945
			63-82	DPYSPSPYGPSPYDR RHAGS	0.755	L7, V8, A9, L10, A11, L12	0.936
			116-125	DRLQGRQQEQ	0.771	L4, T5, I6	0.865
			153-158	GGRDRY	0.635	A16, A17, H18	0.783
			138-151	CGLRSPQRCDLDVE	0.564	Y65, S66, P67, S68, P69, G71, P72, S73, P74, Y75, H79, A80, G81, S82, S83	0.735
			54-60	QREEDQY	0.502	A19, S20, A21, R22	0.722
						G153, G154, R155, R157	0.593
						D147, L148, D149, V150, E151	0.558

A. paraguariensis (*Erectoides*)	85/33	54.8/21.3	1-32	MSKFTILVALALFLL AAHASARQQWELQG DRR	0.805	F4, T5, I6	0.951
			54-61	QRDEDSYE	0.698	L7, V8, A9, L10, A11, L12, F13	0.941
			113-123	DRLQGRQQEQQ	0.687	G71, P72, S73, P74	0.827
			64-77	PYGPSPYGPSPRRA	0.623	H18, A19, S20, A21, R22, Q23	0.819
			91-98	NEFENDQR	0.560	L15, A16, A17	0.705
			132-143	PQQCDLRAPQRC	0.509	A139, P140, Q141	0.699
						Q54, R55, D56, E57	0.698
						P67, S68, P69	0.555
A. macedoi (*Extranervosae*)	92/54	59/34.6	1-29	MSKLTILVALALFLL AAHASARQQWELQG	0.799	L7, V8, A9, L10, A11, L12, F13	0.950
			59-86	QYEQDPYRQDPYDS YDRRHTGSSQHQER	0.683	K3, T5, I6	0.878
			134-156	QQCGLRAPQRCDLDI ESGGRDRY	0.590	Q67, D68, P69, Y70, D71, S72, D74,R75, R76	0.811
			92-95	NEFE	0.556	L15, A16, A17, H18, A19, S20, A21, R22	0.808
			114-121	DRLQGRQQ	0.535	Q59, E61, D63, P64, Y65, R66	0.657
						T78, G79, S80, S81, Q82, H83	0.634
						E26, L27, Q28	0.630
						G118, R119, Q120	0.630
A. dardani (*Heteranthae*)	83/78	51.9/46.3	1-29	MSKLTILVALALFLL AAHASARQQWELQG	0.832	M1, S2, K3, L4, T5, I6, L7, V8, A9, L10, A11, L12, F13, L14, L15, A16, A17, H18, A19, S20, A21, R22, Q23, Q24, W25, E26, L27, Q28, G29, D30	0.818
			115-122	LQGRQQEQ	0.658	A139, P140, Q141, R142	0.782
			55-81	RDEDSYEQDPYGPSP YGPSPRRAGSSQ	0.638	Q54, R55, D56, E57, D58, S59, E61, Q62, D63, P64, Y65, G66, P67, S68, P69, Y70, G71, P72,	0.638

						S73, P74, R75, R76, A77, G78, S79, S80, Q81	
			151-155	GRDRY	0.592	L115, Q116, G117, R118, Q120, E121, Q122, D153	0.619
			135-148	CDLRAPQRCDLDVE	0.59	D144, L145, D146, V147, E148	0.603
A. rigonii (*Procumbentes*)	91/53	58.7/34.2	1-30	MAKLTILVALALFLL AAHASARQQWELQG D	0.811	M1, A2, K3, L4, T5, I6, L7, V8, A9, L10, A11, L12, F13, L14, L15, A16, A17, H18, A19, S20, A21, R22	0.868
			54-82	QRDEDSYEQDPYSPS PYGPSPRRAGSSQH	0.658	D58, S59, Y60	0.744
			113-120	DRLQGRQQ	0.594	G71, P72, S73, P74	0.735
			135-149	CDLRAPQRCDLDVES	0.594	R55, D56, E57	0.695
			91-94	NEFE	0.537	D146, V147, E148	0.680
			151-155	GRDRY	0.512	R75, R76, A77, G78, S79, S80, Q81	0.658
						G117, R118, Q120	0.647
						S66, P67, S68, P69	0.545
						L27, Q28, G29, D30	0.542
A. guarantica (*Trierectoides*)	89/56	55.6/35	1-25	MAKLTILVALALFLL AAHASARQQW	0.818	V8, A9, L10, A11, L12, F13	0.975
			54-86	QRDEDSYEQDPYRQ DPYGPSPYGPSPRRA GSSQ	0.674	M1, A2, K3	0.927
			118-127	DRLQGRQQEQ	0.659	L4, T5, I6, L7	0.881
			140-160	CDLRAPQRCDLDVES GGRDRY	0.590	G76, P77, S78, P79, R80	0.871
						R66, Q67, D68, P69, Y70, G71, P72, S73, P74, Y75	0.812
						G122, R123, Q124	0.746
						L150, D151, V152, E153	0.675
						A19, S20, A21	0.673
						D56, E57, R119	0.642
						R55, D58, S59, E61	0.625
						L120, Q121, Q125	0.575

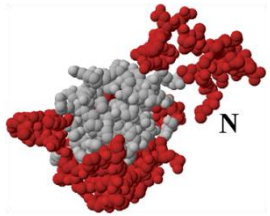
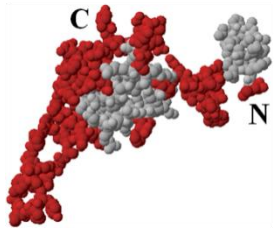
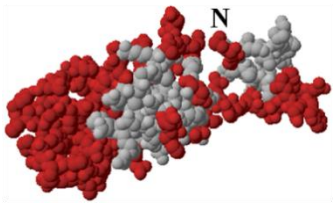
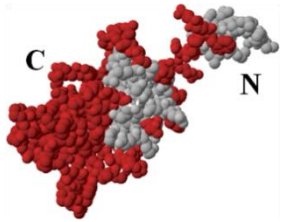
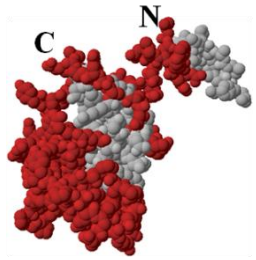
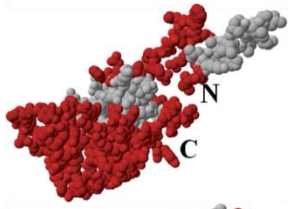
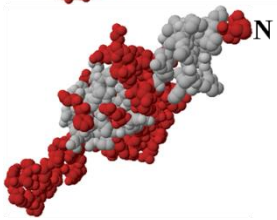
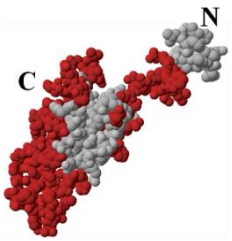
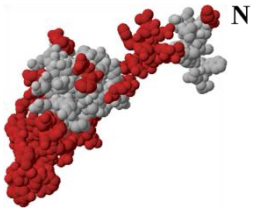
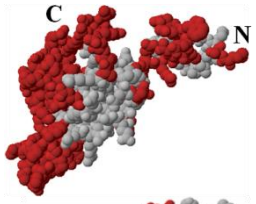
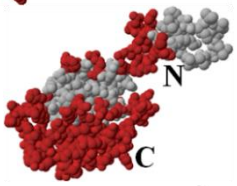
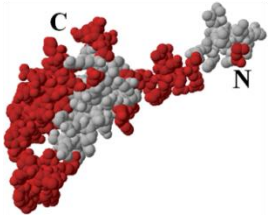
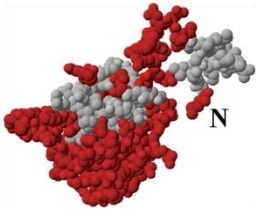
						S154, G155, G156	0.575
						R81, A82, G83, S84, Q86	0.568
A. triseminata (*Triseminatae*)	92/42	58.3/26.6	1-31	MAKLTILVALALLLL AAHASARQQWELQG DR	0.794	V8, A9, L10, A11, L12, L13	0.964
			72-85	YESHDRRRAGSSQH	0.706	A16, A17, H18, A19, S20, A21, R22	0.838
			54-70	QRDQSPYSQDPYRQE PY	0.628	R55, D56, S58, P59, Y60, R117, G120, R121	0.754
			115-126	SDRLQGRQQEQQ	0.597	E73, S74, H75	0.736
			138-146	CGFRAPQRC	0.523	R78, R79, A80, G81, S82, S83, Q84	0.687
			148-151	LEIE	0.522	K3, L4, T5	0.624
			154-158	GRDRY	0.518	S61, Q62, D63, P64, Y65	0.619
						G29, D30, R31	0.549

Table C.2 Conformational epitopes predicted by the DiscoTope server based on the Ara h 2 models. Predictions were made with a threshold value of -7.7.

Species		Residues Included		Conformational Epitopes
		Number	Percent	
A. hypogaea (Ara h 2.01)/ *A. duranensis*		95	59.4%	1M, 21A-32R, 43R, 47Q, 49L-87H, 97-98F, 102Q, 113M-135N, 138Q, 144A-147R, 150L-160Y
A. batizocoi		103	62.4%	1M-2S, 22R-32R, 35S, 39R, 43R, 47Q, 49L-92H, 102E-103F, 107Q, 118M-140N, 143Q, 149A-152R, 155L-165Y

A. glandulifera		102	60%	22R-25W, 28Q-32R, 35S, 39R, 43R, 47Q, 49L-97H, 108F, 110N, 112Q, 124E-145N, 148N, 154A-157R, 160L-170Y
A. ipaensis		109	63.4%	1M, 22R-32R, 35S, 39R, 43R, 47Q, 49L-99H, 109E-110F, 113N-114Q, 125M-147N, 150Q, 156A-159R, 163E-172Y
A. palustris		102	58%	1M-2A, 21A-33C, 35S, 39R, 43R, 47Q, 49L-103H, 114F, 117N, 129M-151N, 154Q, 160A-162Q, 166L-176Y
A. pintoi (*Caulorrhizae*)		95	60.1%	1M-2S, 24Q-32R, 35S, 49L-85H, 95E-96F, 100Q, 111M-130E, 132R-133N, 136Q, 139G, 142S-145R, 148L-158Y

A. paraguariensis (*Erectoides*)		93	60%	1M-2S, 22R-33C, 35S, 39R, 43R, 47Q, 49L-87H, 98F, 101N, 114E-135N, 138N, 144N-147R, 150L-160Y
A. macedoi (*Extranervosae*)		90	57.7%	1M, 21A-33C, 35S, 39R, 43R, 47Q, 49L-83H, 93E-94F, 109M-131N, 134Q, 140A-142Q, 146L-156Y
A. dardani (*Heteranthae*)		93	60%	1M-2S, 22R-33C, 35S, 39R, 35S, 39R, 43R, 47Q, 49L-82H, 93F, 96D, 108M-130D, 133Q, 139A-142R, 145L-155Y
A. rigonii (*Procumbentes)*		91	57.4%	1M, 22R-32R, 35S, 39R, 43R, 47Q, 49L-82H, 92E-93F, 109E-130N, 133E, 139A-142R, 145L-155Y
A. guarantica (*Trierectoides*)		94	58.8%	1M, 21A-32R, 35S, 39R, 43R, 47Q, 49L-87H, 97E-98F, 102N, 114E-135N, 138Q, 141D, 144A-147R, 150L-160Y

A. triseminata (*Triseminatae*)		93	58.9%	1M, 21A-33C, 35S, 39R, 43R, 47Q, 49L-85H, 95E-96F, 111M-133N, 136Q, 142A-144Q, 148L-158Y