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# Assessment of Avenin Polymorphism in Selected Oat Varieties

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# Declaration

I hereby declare that I have done this thesis entitled “Assessment of Avenin Polymorphism in Selected Oat Varieties” independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague 15.4.2020

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Lucie Dostalíková

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# Abstract

Oat (*Avena sativa* L.) is a grain crop from the Poaceae family. Its popularity in human consumption has recorded an increasing trend thanks to the high content of beneficial substances. Oats are also rich in proteins; however, oat prolamins – avenins might be considered as a trigger of toxic effects for patients having celiac disease (CD). Avenins account for 4 – 15% of proteins in the seed. They are heterogeneous, highly polymorphic, and easily separated by SDS-PAGE electrophoresis. With the aim of the avenin band spectra analysis and detailed characterization of avenin polymorphism and varieties relationship, 151 oat varieties of various geographical origins were analyzed by SDS-PAGE by single seed and bulk avenin analysis method. In total, there were detected 26 allelic avenin positions in the molecular weight range of 18 to 35 kDa. The allelic position with the highest occurrence rate was labeled as a band no. 8 (23.5 kDa) and was the same in both types of avenin analyses. Based on the evaluation of the lowest number of bands in celiac reactive peptide spectra (25 – 37 kDa), there are three varieties in each type of avenin analysis that might have the potential to be less reactive and more suitable for patients with celiac CD. All examined samples showed a high level of heterogeneity and high inter-varietal polymorphism since there was no similar avenin band pattern among all of them. Moreover, several samples analyzed by the single seed analysis also showed intra-varietal polymorphism. The dendrogram divided studied samples into six main clusters. The highest diversity in avenin band patterns was detected in varieties from the Czech Republic, whereas the most consistent were Austrian and French varieties. Varieties originating in tropical/subtropical countries showed some similarities with European and Canadian varieties on the level of avenin band spectra. High polymorphism and heterogeneity in the avenin band pattern have potential in easy identification of the variety as well as in a breeding process focusing on the reduction of proteins that are potentially harmful to patients with CD.



**Keywords**: Avenins, celiac disease, oat varieties, SDS-PAGE, seed storage proteins

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# List of the abbreviations used in the thesis

A-PAGE: Acid polyacrylamide gel electrophoresis

AFLP: Amplified fragment length polymorphism

BYDV: Barley yellow dwarf virus

CBB: Coomasie brilliant blue

ccSSR: Consensus chloroplast simple sequence repeat

CD: Celiac disease

DP: Degree of polymerization

FA: Fatty acid

FAO: Food agricultural organization

kDa: Kilodalton

RAPD: Random amplified polymorphic DNA

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

USDA: United States department of agriculture

# 1. Introduction and Literature Review

Oat (*Avena* L.) is a monocotyledon crop from the Poaceae family cultivated almost all over the world in various climatic regions (FAO 2004). Although oat is still predominantly used as forage and livestock feed, the popularity of oat consumption in the human diet recognizes the increasing trend (Ahmad et al. 2010).

From the nutritional point of view, oat can be called as a functional food (Medicine/National Academy of Science 1994). Unlike the other cereals, it is rich in proteins and unsaturated fatty acids (Lásztity 1998). Moreover, oat contains substances with antioxidative activity like avenanthramides, tocopherols, and other phenolic compounds, phytic acid, or sterols (Peterson 2001). Other essential compounds in oats are β-glucans that reduce high blood pressure (Maki et al. 2007), cholesterol levels (Kapur et al. 2008; Ruxton & Derbyshire 2008; Othman et al. 2011) or promote gut health (Malkki & Virtanen 2001; Vasiljevic et al. 2007).

Oat can also be covered in the gluten-free diet for most, but not all patients that are suffering from celiac disease (CD), as its safety was confirmed by research (Peraaho et al. 2004; Kemppainen 2007; Koskinen 2009; Kemppainen 2010). However, oat prolamins – so-called avenins – an alcohol-soluble fraction of oat proteins, are deemed to be the trigger of toxic effects in some CD patients (Lundin et al. 2003; Arentz-Hansen et al. 2004; Tuire et al. 2012).

Avenins account for 4 – 15% of proteins in seed (Lásztity 1998; Klose & Arendt 2012), and they are highly polymorphic. Thanks to this feature, avenins separation can be a very effective, cheap and easily accessible method (Polišenská et al. 2011) for variety identification (Hansen et al. 1988; Gregorova et al. 1996; Dvořáček et al. 2003). High diversity of cultivars is beneficial for the improvement of some valuable traits during the breeding process (Lookhart 1985; Achleitner et al. 2008), and the polymorphic protein pattern can be useful for the reduction of potentially harmful proteins to CD patients (Ahokas et al. 2005).

Thus, the aim of this diploma thesis is to separate avenins of a wide range of oat varieties by SDS-PAGE method, characterize and evaluate allelic avenin polymorphism, assess the intra- and inter-varietal polymorphism on the base of avenin level and geographical origin of each variety, as well as evaluate a relationship among individual varieties.

## 1.1. General Information about Oats

### 1.1.1. Morphotaxonomy

Genus *Avena* L., belonging to the family Poaceae, is quite unambiguous and complicated because it includes species with different ploidy, as well as a vast number of wild species; thus, wide botanical and ecological diversity (Loskutov & Rines 2011). Therefore, the species classification and nomenclature depend on each taxonomist, classification method, or type of analyzed trait (Loskutov & Rines 2011; Boczkowska et al. 2016). The first attempts to create the systematics of *the Avena* genus according to morphological traits started already in the 16th century by Linneaus (1762). Since then, many other types of research have created different taxonomies of this genus.

One of the most detailed descriptions of oat species was done by Russian oat specialist Malzew (1930). He divided the *Avena* genus according to morphological characters (rachilla, lemma, and floret attributes) and biological characteristics into two main subsections – *Aristulatae* Malz. and *Denticulatae* Malz., including altogether seven species and 22 subspecies.

Later, several new species were discovered, and more genetic and cytogenetic data were available; thus, there was a need to establish another classification system. One of the first attempts was made by Mordvinkina (1936), who took into consideration not only morphology but also plant immunology and genetic data as well as the areas of distribution. Mordvinkina´s work was later used together with new genetic, karyological, and morphological data as a base for the creation of a new classification system of the genus *Avena* (Rodionova et al. 1994).

Another essential research was realized by Baum (1974), who included exactly 27 features, such as lodicule and epiblast shapes or ploidy level. The conclusion of his work was an arrangement of the genus *Avena* in seven sections and 34 species (Baum 1977). The modern complex taxonomical system created by Loskutov (2007) is based mostly on historical publications of Malzev (1930), Morvinkina (1936), and Rodionova (1994), including detailed morphology, distribution, ecology and laboratory analysis of oat species.

According to these parameters, genus *Avena* includes 26 species, and it is divided into two subgenera *Avena* L. and *Avenastrum* (C.Koch) Losk. Subgenus *Avena* is divided into two main sections *Aristulatae* (Malz.) Losk. and *Avenae* Losk. (Table 1), while subgenus *Avenastrum* includes one species *A. macrostachya* (Loskutov 2008).

*A. macrostachya* is regarded to be a primitive member of the *Avena* genus (Levitsky 1976; Baum & Rajhathy 1976) originating and occurring only in Algeria (Baum 1977) that differs from *Avena* by its self-incompatibility, perenniality and autotetraploidy ($2n = 28$) (Baum & Rajhathy 1976). Crosses of *A. macrostachya* and cultivated oats are possible (Loskutov 2007). Thanks to its certain traits such as perenniality, rust, aphid, BYDV (Barley yellow dwarf virus) resistance and increased winter hardiness (Leggett 1990; Guarino et al. 1991; Loskutov 2003; Loskutov & Rines 2011), genes of *A. macrostachya* have a potential to be used in oat breeding (Baum & Rajhathy 1976; Pohler & Hoppe 1991).

As mentioned at the beginning of the chapter, a disadvantage of the morphological system is that it might be very variable depending on the keystone trait and sometimes cannot provide complete information about the evolutionary and systematic positions of some species (Loskutov & Rines 2011). Thus, it is advantageous to combine morphological features with biochemical markers such as protein markers (Thomas & Jones 1968; Lookhart & Pomeranz 1985; Loskutov 2007) or isoenzyme analysis (Morikawa 1991; Morikawa 1992; Beer et al. 1993; Hoffman 1996; Guma et al. 2006); and molecular markers, for example, Random Amplified Polymorphic DNA (RAPD-marker) (Loskutov & Perchuk 2000; Drossou et al. 2004), Amplified Fragment Length Polymorphism (AFLP-marker) (Fu & Williams 2008), Consensus chloroplast simple sequence repeat (ccSSR) (Li et al. 2009), and other methods, which give the possibility to overcome these problems (Loskutov & Rines 2011).

**Table 1.** Speciation in the genus *Avena* L. (Loskutov 2007; Loskutov & Rines 2011)

| Section | Species | | | 2n* | Ploidy |
|---|---|---|---|---|---|
| | Wild | | Cultivated | | |
| | Floret disarticulation | Spikelet disarticulation | | | |
| *Aristulatae* (Malz.) Losk. | *A.clauda* Dur. | *A.pilosa* M.B. | | 14 | 2 |
| | *A. prostrata* Ladiz. | | | | |
| | *A.damascena* Raj.et Baum | | | | |
| | *A. longiglumis* Durie. | | | | |
| | *A. wiestii* Steud. | *A. atlantica* Baum | | | |
| | *A. hirtula* Lagas. | | *A. strigosa* Schreb. | | |
| | *A. barbata* Pott. | | | 28 | 4 |
| | *A. vaviloviana* Mordv. | | *A. abyssinica* Hochst. | | |
| *Avenae* Losk. | | *A. ventricosa* Bal. | | 14 | 2 |
| | | *A. bruhnsiana* Grun. | | | |
| | | *A. canariensis* Baum | | | |
| | | *A. agadiriana* Baum et Fed. | | 28 | 4 |
| | | *A. magna* Mur. et Terr. | | | |
| | | *A. murphyi* Ladiz. | | | |
| | | *A. insularis* Ladiz. | | | |
| | *A. fatua* L. | *A. sterilis* L. | *A. byzantina C.* Koch | 42 | 6 |
| | *A. occidentalis* Durie. | *A. ludoviciana* Durie. | *A. sativa* L. | | |

* Number of chromosomes in somatic cell

### 1.1.2. Origin and Distribution

Oat is one of the youngest cultivated cereals in the world because its domestication started much later than in wheat or barley (Murphy & Hoffman 1992), probably no earlier than 4,000 years ago (Zohary & Hopf 1988). As it is shown in Table 1, several main oat species are cultivated these days. *A. sativa* – common oat has the most significant importance in world grain production. There are two forms – hulled one (*A. sativa* subsp. *sativa*), originating in Iran, Georgia, and Russia – and hull-less form (*A. sativa* subsp. *nudisativa*), originating in Mongolia and China (Loskutov 2008).

*A. nuda* – naked oat or hull-less oat is originating in the United Kingdom (Loskutov 2008) and has lower economic importance than *A. sativa*. Hexaploid species, such as *A. byzantina* that is considered being a native species in Algeria and Morocco, and *A. abyssinica* – Ethiopian oat, are both rather minor crops (National Research Council 1996). The last one, *A. strigosa* or lopsided oat, bristle oat, or sand oat, is grown mainly for fodder (Fontaneli et al. 2012; Diederichsen 2014) and originates in Spain and Portugal (Loskutov 2008).

## 1.2. Cultivation and Production of Oats

*A. sativa is* produced on the six continents of the world as a crop used for human food and animal feed. Generally, the world annual oat production and the associated harvested area have been decreasing till 2010, but since then, both items have a slightly increasing trend (FAO 2017).

Oats are very well adapted to colder and moist climates, but most of the important oat species do not tolerate very hot, dry, or very humid weather; therefore, oat cultivation is concentrated, preferably in temperate climatic zones (FAO 2004). Countries occupying the highest positions in world oat production are generally located in a temperate climate such as the Russian Federation, Canada, Australia, Poland, and China, mainland. In 2017, the highest numbers for the production of oat were reached in Europe (over 14 million tonnes). However, oat is also cultivated in subtropical and tropical countries such as Brazil, Peru, Ethiopia, Turkey, as can be seen in Figure 1.

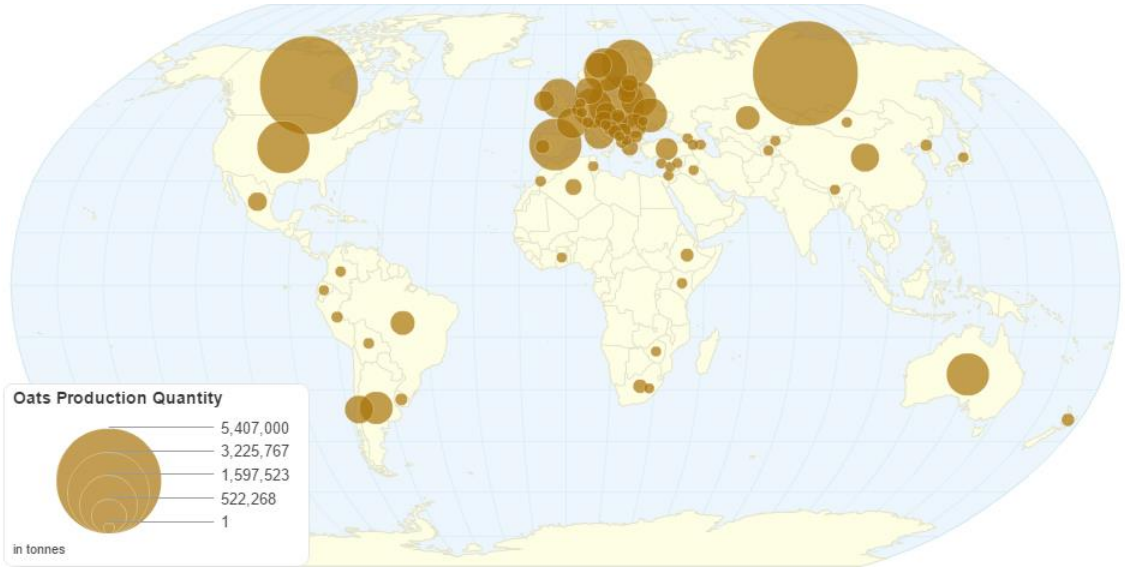


**Figure 1.** Map of world oat production in tonnes per the year 2017 (ChartsBin 2017)

In Latin America, the area of oat fields and annual production is significantly increasing due to the wide adoption of the no-till system by many farmers, where oats play a crucial role in rotation with other crops. Economically essential species – *A. sativa* and *A. byzantina* were introduced to Latin America from Europe as well as species with a low economic value like *A. strigosa*. There are two main tropical/subtropical regions in South America, where oat can be grown – Brazil and the Andean region, which includes Peru, Bolivia, Colombia, and Ecuador (FAO 2004).

South region of Brazil with a humid subtropical climate (Köppen 1936) is characterized by medium fertile soil with a high amount of aluminum (Hudson 1997). Oat is grown mainly in the winter season. It is a popular crop because of low seed and production cost, good aluminum tolerance, adaption for poor soil, and root disease resistance. It also helps with soil improvement. In tropical regions of Brazil, environmental conditions are quite similar to subtropical ones – oat is also sown during winter months with the lowest humidity and rainfalls. In both areas, the major problem is the crown rust but also frost or drought damage (FAO 2004).

Cultivation of oat in the Andean region seems to be more complicated because of high elevation (over 2,600 m above sea level) and quite a long vegetative period – up to 7 months to complete full maturation. There is also a stem rust issue during more humid months (FAO 2004). According to FAO (2017), the highest annual oat production in the regions mentioned above (besides those with temperate climate) was achieved in Brazil, where the value of production has reached over 636 thousand tonnes. In contrast, the lowest production was detected in Ecuador – only 885 tonnes.

In the Middle East region, the oat production is relatively low except Turkey, with the production of 250 thousand tonnes in 2017 that makes this country the most important producer in this area (FAO 2017).

Northwest African region or the so-called Maghreb is a region including countries like Algeria, Morocco, Tunisia, Libya, and Mauritania that are bordering with the Mediterranean Sea. Eighty-seven percent of the area is covered by desert Sahara, unfavorable for agricultural activities. The only suitable agricultural area is located in the coastal belt with a typical subtropical Mediterranean climate (Alterra 2012). Algeria, with the production of 64 thousand tones in 2017 (FAO 2017), is the best oat producer in the Maghreb and in the whole African continent.

In Ethiopia and Kenya, oats are predominantly grown in higher elevations (e.g., Ethiopian highlands) for grain, fodder, or hay (FAO 2004). At lower altitudes, they are less adapted and therefore have been replaced by grass sorghum (Boonman 1993). According to FAO, in 2017, the oat production in Ethiopia has reached only approximately 46 thousand tonnes (FAO 2017).

Generally speaking, total oat production in Africa is low compared to the most prominent world producers located in temperate zones. For example, in 2017, the total oat production did not exceed even 5% of European production (FAO 2017). The factors playing the crucial role in this difference are, of course, climate conditions, but also the level of development of agricultural techniques and breeding programs (Boczkowska et al. 2016).

## 1.3. *Avena sativa* Morphology

The common oat (*A. sativa*) is annual grass 40 – 180 cm high with erect, semi-erect, or prostrate stem and very deep fibrous root system. The number of tillers oscillates between 3 to 5 per plant. As with all cereals, the stem is divided into nodes and internodes (Assefa 2006; Tiwari 2010).

Simple linear leaves are 8 – 45 cm long; dark green with high ligule (2 – 8 mm long) and no auricles, alternately arranged and left rotated with acuminate leaf-blade apex (Figure 2) and scaberulous surface (Clayton et al. 2006). The bottom part of the leaf blade is usually covered by longer trichomes, in contrast to long and loose leaf sheath, which is usually without hairs. The texture of the leaf blade is generally rough and sandpapery due to tiny prickles (Assefa 2006; New England Wild Flower Society 2011).

Branched inflorescence – terminal panicle – is formed by rachis and pedicels holding uniform spikelets with two fertile florets in each spikelet. Florets basically contain stigma and three anthers surrounded by lemma and palea remaining attached at maturity. Lemma is typically not hairy, and it could sometimes have an awn on its end emerging closely from the base (Wu & Philips 2006; Clayton et al. 2006). The lemma ranges in color from white, yellow, grey, and red to black. The spikelet is always surrounded by glume, which never has an awn (Stanton 1953). The fruit is a yellow to brown spiky caryopsis (or groat) with visible groove, (linear hilum) running on from the

top to the bottom of the seed. It is covered by a removable hull consisting of lemma and palea (Assefa 2006). There are tiny trichomes on the surface of the seed with the highest concentration on the top of the seed (Arendt & Zannini 2013). The seed itself consists of three main parts – bran or pericarp, endosperm with aleurone layer, and embryo (Figure 3).

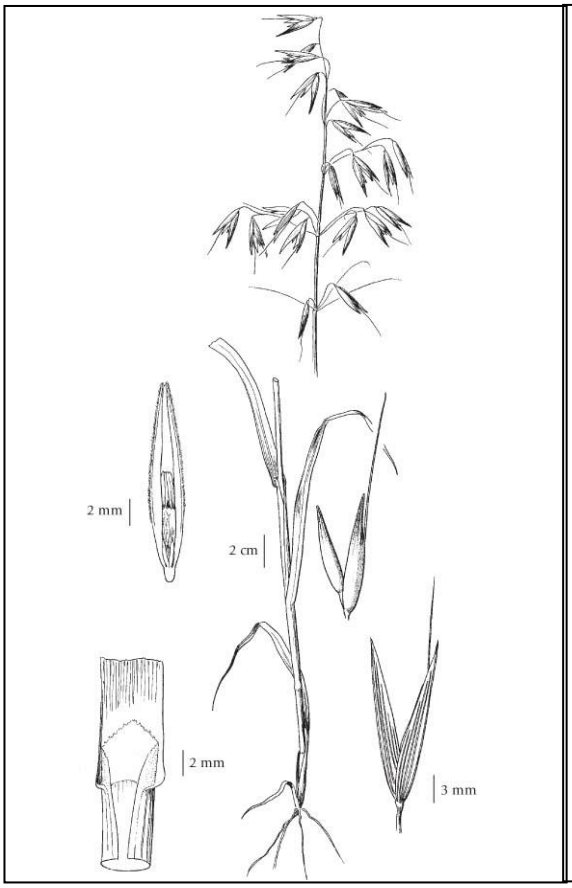


**Figure 2.** *Avena sativa* plant (Douglas et al. 2001)

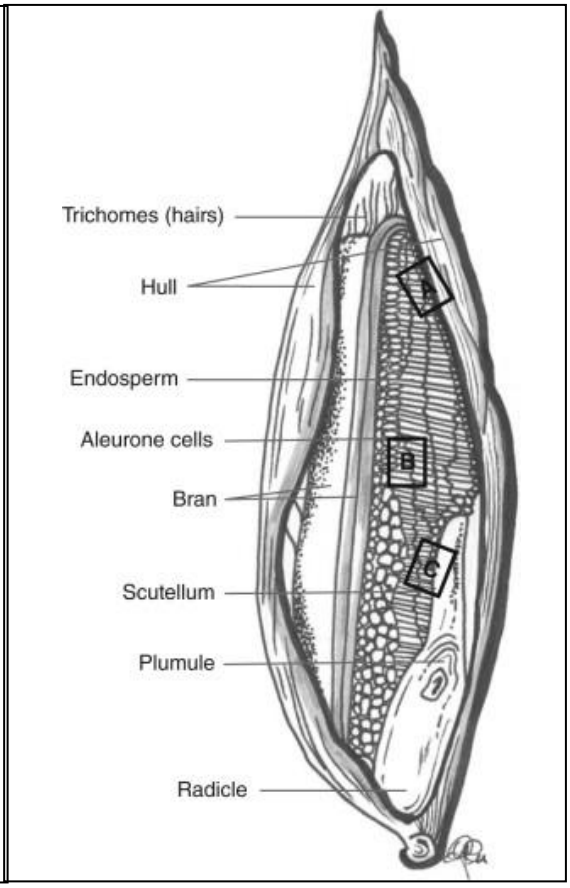


**Figure 3.** Oat caryopsis. A – bran or pericarp, B – endosperm with aleurone layer, C – endosperm (Arendt & Zannini 2013)

### 1.3.1. Oat in Human Diet

Although oats have been grown from ancient times primarily as animal feed, they became an accepted part of the human diet over the 19th century (Sahlstrøm & Knutsen 2010). However, the popularity of oats started to increase thanks discovery of the excellent nutritional value of grains and many health benefits and effects (Ahmad et al. 2010). Therefore, oat could be called as a functional food – a food providing the health benefit beyond the traditional nutrients it contains with it (Institute of Medicine/National Academy of Science 1994).

#### 1.3.1.1. Oat Based Foods

Oat can be found in a wide variety of commercial products that are available on the market, such as bake products, cold cereals, oat flour, meat product extenders, baby food, granolas and snack, oat ice creams, pancake mix, and coffee substitute (Lim 2013) but the most extensive use of oats is as a breakfast cereal. During the cooking process, starches start to gelatinize and form a biphasic paste – so-called porridge (Tecante & Doublier 1999).

By oat fermentation, milk-type (oat milk) and yogurt-type products can be obtained, which can serve as a substitute of milk for milk allergenic or lactose intolerant patients (Lindahl et al. 1997; Onning et al. 1998; Sontag-Strohm et al. 2008).

Oats can also be used in bake products, although it does not have such excellent properties for baking as wheat due to lower gluten content that gives the dought viscoelastic properties (Oomah 1983). Up to 5 – 20% of wheat flour can be replaced with oat flakes in traditional wheat bread processing (Gormley & Morrissey 1993). Flander et al. (2007) successfully optimized baking process and ingredients ratio for 51 – 100% oat bread by adjusting water and added gluten content, resulting in a high level of β–glucans and still keeping good sensory attributes of the final product that has, comparing to wheat bread, better nutritional and biological value due to higher protein content and favorable amino acids balance (Hahn et al. 1990; Gambus et al. 2011). Moreover, the oat protein quality is almost as high as soy protein, which is, according to the World Health Organisation, equal to meat, milk, and egg proteins (Lásztity 1999).

Oat has also been used in several traditional dishes like Scottish “sowan” made by oat starch thickening (McNeill 1929), British oatmeal caudle made from oatmeal, spices, and ale or in the cold beverage made from ground oat and milk, traditional in Latin America (Lim 2013).

### 1.3.1.2. Nutritive Properties

Oat caryopsis could be divided into three main parts – bran (38 – 40% of caryopsis weight), endosperm (58 – 60% of caryopsis weight), and embryo (3% of caryopsis weight), with variable distribution of nutrients in each of the main parts. When comparing oat bran composition to the other cereals such as wheat, oat typically has a higher content of fats, proteins and carbohydrates (Table 2), as well as soluble fiber (β-glucans), and antioxidants (Lásztity 1998).

**Table 2.** Comparison of proximate nutrients of oat (*A. sativa*) and wheat (*Triticum aestivum* L.) bran in dry matter (USDA 1989a, b)

| **Proximate nutrients of oat bran** | **Value per 100 g** | **Proximate nutrients of wheat bran** | **Value per 100 g** |
|---|---|---|---|
| Water | 6.55 g | Water | 9.89 g |
| Energy | 246 kcal | Energy | 216 kcal |
| Protein | 17.3 g | Protein | 15.5 g |
| Fat | 7.03 g | Fat | 4.25 g |
| Carbohydrates | 66.22 g | Carbohydrates | 64.51 g |
| Dietary fibre | 15.4 g | Dietary fibre | 42.8 g |

**Starch**

Starch is the primary representative of carbohydrates in oat caryopsis, occurring predominantly in endosperm as irregular, tetrahedral, even ovoid or hemispherical (Jane et al. 1994; Hoover et al. 2003; Tester et al. 2004) and relatively small granules (Hoseney et al. 1971; Hoover et al. 2003), which often form clusters (Matz 1969). Its level oscillates between 43.7% – 61% (Paton 1977), or according to newer research, around 50 – 60% (Sayer & White 2011), depending on the variety or growing season.

Oat starch is composed of non-carbohydrate and carbohydrate elements. Although non-carbohydrate components occupy only minor levels (1 – 2% of starch) (Tester et al. 2004), they can strongly affect the physicochemical properties of starch (Buleon et al. 1998; Zhang & Hamaker 2003).

The perfect examples of non-carbohydrate components are lipids that are present in the form of amylose-lipid complex, whose levels are higher than in wheat, corn, or rice starch (Sowa & White 1992; Zhou et al. 1998). The number of lipids captured in the amylase-lipid complex may positively influence rheological properties or starch retrogradation (Gudmundsson & Eliasson 1989). On the other hand, they may reduce water-binding capacity, swelling, starch solubilization, or cause unfavorable flavor and taste due to oil rancidity. Proteins presented in oat starch reach only low levels, but unlike oils, they mostly have adverse effects like a mealy flavor or foam building (Swinkels 1985).

Amylose and amylopectin – carbohydrate constituents influence starch features by their ratio (Hoover & Vasanthan 1994), chain length distribution, or spacing of branch points on the amylopectin molecule (Tester & Morrison 1990, Jane et al. 1999). Amylose is characterized by a low degree of polymerization (DP) (3000) and a branching frequency with low α-1,6 linkage frequency, while amylopectin tends to have high DP (> 5000) and higher α-1,6 linkage frequency (Bruce & Matthew 2009).

**Dietary Fiber**

Oat grain contains 10.2 – 12.1% of fiber, and its main components are non-starch indigestible polysaccharides that can be analytically split into water-soluble and water-insoluble substances. The water-soluble polysaccharides are represented by gums, mucilages, pectins, some hemicelluloses, arabinoxylans, and very important β-glucans. Lignin, cellulose, and resting hemicelluloses are the components of the insoluble fiber (Manthey et al. 1999).

The importance and health benefits of dietary fiber in the human diet are significant. Lower absorption of glucose and sterols by the intestine (Kahlon & Chow 1997), reducing intestine transit, or delaying gastric emptying (Anderson & Bridges 1988) are examples of positive effects of water-soluble dietary fiber.

**β-glucans**

β-glucans are linear unbranched polysaccharides consisting of a mixture of 1-4 and 1-3 linked β-D-glucopyranose units, predominantly present in starchy endosperm and subaleuron region. The reported amount ranks between 3 – 7% (Wood 1992) depending on oat cultivar, environment (Ames et al. 2006; Havrlentová 2009) and developmental stage of the seed, as their amount is reduced during germination (Mikola & Jones 2000), but cultivation method or nitrogen fertilization does not play any role in β-glucans levels (Saastamoinen et al. 2004). Comparing to other cereals, the only crop more abundant in β-glucans is barley (Wood 1992).

β-glucans play key role in the human diet, because of several medicinal properties such as reduction of blood pressure and ischemic heart diseases (Maki et al. 2007) as well as lowering the cholesterols levels by inhibiting the absorption of dietary cholesterol in intestine and reabsorption of bile acids produced by the liver (Kapur et al. 2008; Ruxton & Derbyshire 2008; Othman et al. 2011). They also work as prebiotics and promotes intestine health (Malkki & Virtanen 2001; Vasiljevic et al. 2007). Bacterial fermentation also transforms β-glucans into short-chain fatty acids that are able to inhibit hepatic cholesterol synthesis (Rondanelli et al. 2009). According to Cheol-Heui et al. (2003), β-glucans may help to increase resistance to infections caused by *Staphylococcus aureus* and *Eumeria vermiformis* in mice or promote immunological activity (Estrada et al. 1997).

**Lipids**

Lipids are predominantly present in the embryo (highly in scutellum), as well as in the starchy endosperm or oat bran (Youngs et al. 1977), whereas the lowest oil concentration is in the hull. They can be captured in amylose-lipid complex, attached to starch granules surface as surface lipids, or remain in the free state as non-starch lipids. The oil amounts may reach various levels (approximately from 2 – 11.6%) according to oat variety (Brown & Craddock 1972; Frey & Hammond 1975), method of oil content determination (Zhou et al. 1999) and level of processing (Arendt & Zanini 2013). The total oat lipid content can be divided into several fractions – triglycerides that reach the highest values around 32 – 85%, then phospholipids, glycolipids, free fatty acids, and sterols (Lásztity 1998).

The nutritional quality of oat seed oil is excellent because of the predominance of unsaturated fatty acids that are beneficial for human health (Tapiero et al. 2002; Lunn & Theobald 2006), but the fatty acid (FA) composition may alter in different cultivars (Table 3). According to Brindzová et al. (2008), the primary FA is monounsaturated oleic acid (18:1), followed by polyunsaturated linoleic acid (18:1) and saturated palmitic acid (16:0) (Table 3), which gives rise to a relatively low melting point (Lásztity 1998). Similar variations in fatty acids composition of different oat cultivars were also discovered previously (De la Roche et al. 1977; Saastamoinen et al. 1989).

**Table 3.** Fatty acid composition of four *A. sativa* cultivars (data expressed on a dry weight basis) (Brindzová et al. 2008)

| | Fatty acids (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Cultivar** | **14:0** | **16:0** | **16:1** | **18:0** | **18:1 - 9c** | **18:1 - 11c** | **18:2** | **18:3** | **20:1** |
| Detvan | 0.3 | 17.2 | 0.2 | 1.9 | 38.2 | 1.0 | 36.8 | 1.2 | 0.8 |
| Jakub | 0.2 | 15.5 | 0.2 | 2.1 | 42.2 | 0.8 | 35.4 | 1.3 | 0.8 |
| Izak | 0.3 | 16.2 | 0.2 | 1.6 | 38.7 | 1.0 | 38.1 | 1.3 | 0.8 |
| Triumph | 0.4 | 16.6 | 0.2 | 2.1 | 35.6 | 0.9 | 37.3 | 1.2 | 0.8 |

However, the disadvantage of oat oil is the presence of free fatty acids. The value ranges between 2 – 11% of the total lipid content in endogenous grain, which is not enough to cause any adverse effects (Zhou et al. 1999), but its levels rapidly increase during inappropriate processing and storage conditions, causing rancidity and off-flavors in the product (Welch 1977; Ekstrand et al. 1992; Molteberg et al. 1995).

## Other Components

Oat is relatively rich in mineral components such as P, K, Ca, Mg, and Na, from the trace elements, Fe, Zn, Mn, and Cu are present. Comparing the major mineral composition of other cereals; oat contains more K, P, Mg and Ca (Welch et al. 2011) and the levels of minor components are relatively higher (Table 4); however, their amount is strongly affected by their availability in soil (Welch et al. 2011). The content of vitamins also varies compared to other cereals, but oat generally contains more thiamine, biotin, and choline. On the other hand, it is low in niacin and vitamin B6 (Welch 2006).

**Table 4.** The mineral content of cereal grains (U.S. Department of Agriculture, Agricultural Research Service 2008; Welch 2006; Welch 2011)

| mg/100 of fresh weight | Whole-Grain Oatmeal | Whole-Grain Wheat | Whole-Grain Cornmeal | Brown Rice | Whole-Grain Rye | Pearled Barley | Sorghum |
|---|---|---|---|---|---|---|---|
| Potassium | 389 | 373 | 319 | 247 | 337 | 286 | 318 |
| Phosphorus | 459 | 333 | 266 | 302 | 367 | 242 | 289 |
| Magnesium | 145 | 129 | 134 | 127 | 107 | 80 | 156 |
| Calcium | 54 | 36 | 12 | 22 | 32 | 24 | 28 |
| Sodium | 9 | 4 | 38 | 4 | 3 | 5 | 15 |
| Iron | 4.3 | 3.9 | 3.2 | 1.6 | 2.7 | 2.7 | 4.8 |
| Zinc | 3.4 | 2.9 | 1.9 | 1.9 | 3.4 | 2.1 | 2.2 |
| Manganese | 4.1 | 3.5 | 0.6 | 3.0 | 1.7 | 1.2 | 1.8 |
| Copper | 0.44 | 0.42 | 0.3 | 0.56 | 0.44 | 0.39 | 0.98 |

Except for β-glucans, oats also contain other useful compounds such as vitamin E (tocopherols, tocotrienols), phenolic acids, flavonoids, and 24 unique avenanthramides, which are present only in oats (Collins 1989; Collins 2011). Those compounds can be classified as phenolics, and together with sterols, phytic acid and flavonoids that are also present in oat grain have antioxidative activity. Therefore, they are not beneficial only for human health but also as a protection of food products from rancidity, promoting more extended shelf life, stability, and taste (Peterson 2001).

Moreover, avenanthramides are considered to have antiatherogenic activity (Nie et al. 2006a; Nie et al. 2006b; Koenig et al. 2011), anticancer activity (Hastings & Kenealey 2017; Scarpa et al. 2018); anti-inflammatory properties (Liu et al. 2004; Guo et al. 2008; Sur et al. 2008) and antimicrobial effects – they are so-called phytoalexins (Dimberg & Peterson 2009).

## 1.4. Oat Proteins Description

As mentioned above, oats contain higher levels of proteins, and as a benefit, its protein content has higher biological value due to the presence of lysine – essential amino acid that is a limiting factor in other important cereals like wheat or maize (Pomeranz et al. 1973). On the other hand, glutamic acid (glutamine) and proline contents are relatively lower (Lásztity 1998). The part that is the richest in protein bodies is the germ (29 – 38%), followed by bran (18 – 26%) and endosperm (12%) (Youngs 1972; Lásztity 1998; Miller & Fulcher 2011).

Seed proteins can be divided into four categories according to Osborne´s classification (Laemmli 1970) based on their solubility: albumins, glutelins, prolamins, and globulins (Table 6). The last two mentioned are the significant fractions of oat caryopsis (Robert et al. 1983; Rober et al. 1985), unlike Triticeae cereals with a major alcohol-soluble fraction of prolamins (Lásztity 1998).

The amino acid composition of oat proteins may vary on the level of oat variety (Youngs 1972), groat fraction (Fulcher 1986), or protein fractions - the lysine content is higher in globulin fraction, whereas prolamin fraction is rich in glutamic acid. The amino acid composition is not affected by nitrogen, phosphorus, or potassium fertilization (Youngs et al. 1982), but the deficiency of mentioned elements may affect the protein quality significantly (Eppendorfer 1978). The overall amino acid composition of groats from 289 analyzed oat cultivars is given in Table 5.

**Table 5.** Average amino acid composition of groats in 289 cultivars of *A. sativa* (Pomeranz et al. 1973)

| Amino Acids | Value per 100 g | Amino Acids | Value per 100 g |
|---|---|---|---|
| Glutamic acid | 23.9 g | Serine | 4.2 g |
| Aspartic acid | 8.9 g | Lysine | 4.2 g |
| Arginine | 6.9 g | Threonine | 3.3 g |
| Glycine | 4.9 g | Tryptophan | 0.23 g |
| Leucine | 7.4 g | Isoleucine | 3.9 g |
| Proline | 4.7 g | Cystine | 1.6 g |
| Valine | 5.3 g | Tyrosine | 3.1 g |
| Phenylalanine | 5.3 g | Histidine | 2.2 g |
| Alanine | 5 g | Methionine | 2.5 g |

## Oat Globulins

Globulins – salt soluble fraction – account for about 75% of total seed protein (Colyer and Luthe 1984; Lásztity 1998) (Table 6). For globulin analysis, the sedimentation coefficient can be used. It is defined as a ratio of sedimentation velocity of the molecules/particles to the acceleration applied to cause sedimentation. Thus, from the total globulins, three fractions with sedimentation coefficients of 3S, 7S, and the most frequent 12S can be separated (Burgess et al. 1983; Shotwell 1999).

12S globulin is organized in hexamers (Walburg & Larkins 1983) and mostly occurs in the endosperm, unlike 7S globulin that is somewhat localized in the embryo (Burgess & Miflin 1985). Besides, 12S globulin has similar polypeptide subunits organization as 11S legume globulins like soy glycinins (Peterson 1978; Brinegar & Peterson 1982), but their secondary and tertiary structure gives them different properties, such as structural stability at alkaline pH (Marcone et al. 1998; Marcone 1999) and higher thermal denaturation temperature (Harwalkar & Ma 1987; Gorinstein et al. 1996).

## Oat Prolamins - Avenins

Prolamins (avenins) belong to the group of alcohol-soluble polypeptides (Lásztity 1998), relatively rich in proline and glutamine (Shewry et al. 1999; Klose & Arendt, 2012). Avenins are associated with the elicitation of coeliac disease (Armstrong et al. 2012), but compared to wheat, rye, and barley, proline and glutamine levels in oat are rather low (Comino et al. 2011).

The avenin fraction in oat amounts 4 – 15% of the total seed protein (Table 6), depending on cultivar, growth conditions, and extraction method (Lásztity 1998; Peterson 2011, Klose & Arendt 2012). Oat prolamins are mostly present in the endosperm but not in the aleurone layer (Lending et al. 1989; Klose & Arendt 2012).

Avenins show high polymorphism within and between genotypes, probably due to encoding by multigene families. The amount of avenin coding sequences is up to 25 per haploid genome in the case of *A. sativa* (Chesnut et al. 1989), but this number may vary among different cultivars due to, for example, gene duplication (Londono et al. 2013).

For avenin analysis, the electrophoretic separation can be used. Avenins yield different band patterns than wheat gliadins, generally with fewer bands, that move in the molecular weight range of 20 – 36 kDa (Dvořáček et al. 2003), 20 – 40 kDa (Shewry 1999), 25 – 35 (Mickowska et al. 2015) or 14 – 35 kDa (Benoit et al. 2017). The reported molecular weights may vary according to analyzed cultivars or method.

The structure of oat prolamin is expected to be formed by nonrepetitive sequences of α-helix-rich globular structures, and β-reverse turns – proline-rich repeats in two separate blocks, similar to *Triticeae* prolamins (Shewry et al. 1999). The analysis of N-terminal sequence shows an apparent homology among avenin polypeptides as well as between avenins and prolamins of wheat, rye and barley (Shotwell et al. 1990), but low content of proline and high content of leucine and valine corresponds more to millet, rice and maize prolamins (Lásztity 1996).

**Minor Protein Fractions**

Water-soluble albumins account for about 1 – 12% of total seed protein in oat (Table 6). This fraction mostly contains metabolically active proteins such as enzymes like maltase, α-amylase, lichenase, phosphatase, tyrosinase, antinutritive compounds like proteinase inhibitors, trypsin and chymotrypsin inhibitors and several structural proteins (Klose & Arendt 2012; Lásztity 1998). Albumin fraction contains higher amounts of lysine – unlike other oat fractions – asparagines and alanine, but there are quite low levels of glutamines (Peterson & Brinegar 1986).

Oat glutelin is a residue protein that can be extracted with basic or acidic solution after removal of the other free fractions; however, the extraction is not always complete so that some nitrogen compounds may remain as residues. Its amount in total seed protein is lower than 10% (Weiser et al. 1980; Lásztity 1998).

**Table 6.** Division of oat protein fractions according to Osborne (Ma & Harwalkar 1984; Lásztity 1998; Shewry 1999, Benoiet et al. 2017; Peterson 2011; Klose & Arendt 2012)

| Osborne class | Protein (function) | Molecular weight (kDa) | % of protein |
|---|---|---|---|
| Globulins | 12S | 53 – 58 | 70 – 80 |
| | 7S | 50 – 70 | – |
| | 3S | 48 – 52 | – |
| Prolamins | Avenins | 14 – 40 | 4 –15 |
| Albumins | Various | 14 – 17, 20 – 27, 36–47 | 1 – 12 |
| Glutelins | Unextracted globulins and prolamins, minor polypetides | – | < 10 |

## 1.5. Oat Sensitivity and Suitability for Celiac´s Diet

Celiac disease is an autoimmune enteropathy triggered by the ingestion of gluten proteins – prolamins – from cereals (Rostom et al. 2006; Kagnoff 2007; Gregor et al. 2010) causing inflammation of the small intestine and villous atrophy affecting circa 1% of the Western population (Dubé et al. 2005; Mustalahti et al. 2010), but unfortunately, recent studies suggest that its trend is increasing (Ludvigsson et al. 2013). In oats, these prolamins are called as avenins and account for 4 – 15% of proteins in seed (Lásztity 1998; Klose & Arendt 2012).

Many celiac organizations had not supported the recommendations about oat consumption until 1995 (Thompson 2003), when Janatuien et al. (1995) published a study about safe oat consumption in moderate amounts (circa 50 – 70 g/day), concluding no harmful effect on adults with CD. Since that, changes in recommendations related to oat consumption have been made (Thompson 2003), because many scientific studies and trials also confirmed oat suitability for CD patients (Srinivari et al. 1996; Reunala et al. 1998; Picarelli et al. 2001; Thompson 2003; Peraaho et al. 2004; Kemppainen 2007; Koskinen 2009; Kemppainen 2010; Kaukinen et al. 2013).

On the other hand, several studies suggested that oat consumption may induce toxic effects in some CD patients (Lundin et al. 2003; Arentz-Hansen et al. 2004; Tuire et al. 2012). These different results could be explained by the fact that variety of studied oats was ignored (Silano et al. 2007; Comino et al. 2011; Real et al. 2012; Silano et al.

2014) or the purity of used oat was not sufficient (Dickey 2008; Ellis & Ciclitira 2008; Gelinas et al. 2008; Thompson 2004).

Currently, oat is defined as a food tolerated by most but not all CD patients, so it is included, with some regulations, among the gluten-free ingredients by several European Regulations (Commission Implementing Regulation 828/2014 (2014), Commission Regulation 41/2009 (2009)). It is also recommended for consumption by many international organizations, such as Society for the Study of Celiac Disease, Coeliac Disease Foundation, World Gastroenterology Organisation, Coeliac UK, or Canadian Celiac Association.

# 2. Aims of the Thesis

The main objective of the diploma thesis was to analyze and determine the allelic variability of avenins from *A. sativa* germplasm of various geographical origins.

**The sub-objectives were to:**

- Identify allelic variability of avenins in single seed samples
- Identify allelic variability of avenins in bulk samples
- Characterize intra- and inter-varietal polymorphism of avenins
- Evaluate position frequencies of each allele
- Describe the differences in individual avenin profiles

**Hypothesis:**

I. There is no difference in allelic variability of avenins analyzed by single seed and bulk samples method.

II. There is an avenin polymorphism among oat cultivars.

III. The geographical origin of samples affects the avenin variability.

# 3. Materials and Methods

## 3.1. Plant Material

A total of 151 oat varieties originating from 20 different countries across Europe, North and South America, Africa, Asia, and Australia were involved in the study. The seeds of 141 oat varieties were provided by breeding company Selgen, a.s., Krukanice, Czech Republic. Ten varieties were obtained from Gene Bank, Crop Research Institute, Czech Republic.

**Figure 4.** Oat collection on the experimental field in the year 2018 in Selgen a.s., Krukanice, Czech Republic (Hlásná Čepková 2018)

In total, 127 samples harvested in 2017 were subjected to the avenin analysis by a single seed method, and 151 samples harvested in 2018 were analyzed by the bulk method. The difference in the number of analyzed seeds was due to the impossibility of harvesting of all varieties in 2017. The remaining samples were delivered after it was found that the better results were obtained through bulk analysis, and therefore the later supplied samples were not subjected to the single seed analysis.

**Table 7**. Experimental materials from Selgen a.s., Czech Republic

| No. | ECN | Name of variety | Type | Country of origin | No. | ECN | Name of variety | Type | Country of origin |
|---|---|---|---|---|---|---|---|---|---|
| 1 | As18001 | Abel | naked | CZE | 20 | As18021 | Český žlutý | husked | CZE |
| 2 | As18002 | AC Preaknes | husked | CAN | 21 | As18022 | Dakar | black | FRA |
| 3 | As18003 | Adam | naked | CZE | 22 | As18023 | Dalimil | husked | CZE |
| 4 | As18004 | Apollon | husked | RUS | 23 | As18024 | David | husked | CZE |
| 5 | As18005 | Ardo | husked | CZE | 24 | As18025 | Debyut | husked | BEL |
| 6 | As18006 | Atego | husked | CZE | 25 | As18026 | Diadém | husked | CZE |
| 7 | As18007 | Auron | husked | CZE | 26 | As18027 | Dominik | husked | GER |
| 8 | As18008 | Avenuda | naked | CZE | 27 | As18028 | Drummer | husked | GER |
| 9 | As18009 | Avesta | black | FRA | 28 | As18029 | Dukat | husked | POL |
| 10 | As18010 | Azur | husked | CZE | 29 | As18030 | Ebene | black | FRA |
| 11 | As18011 | Belinda | husked | FIN | 30 | As18031 | Efendi | husked | AUT |
| 12 | As18012 | Bison | husked | RUS | 31 | As18032 | Espresso | husked | AUT |
| 13 | As18013 | Black | black | FRA | 32 | As18033 | Expander | husked | AUT |
| 14 | As18014 | Buggy | husked | GER | 33 | As18034 | Expo | husked | AUT |
| 15 | As18015 | Cavaliere | black | CZE | 34 | As18035 | Flamingsnova | husked | GER |
| 16 | As18016 | CC4146 | black | GBR | 35 | As18036 | Flämingsprofi | husked | GER |
| 17 | As18017 | CDC Boyer | husked | CAN | 36 | As18037 | Florian | husked | CZE |
| 18 | As18018 | Classic | husked | USA | 37 | As18038 | Freddy | husked | GER |
| 19 | As18019 | Coach | husked | GER | 38 | As18039 | Gana | naked | RUS |

**Table 7.** Continued

| No. | ECN | Name of variety | Type | Country of origin | No. | ECN | Name of variety | Type | Country of origin |
|---|---|---|---|---|---|---|---|---|---|
| 39 | As18020 | Cyril | husked | CZE | 59 | As18040 | Garland | husked | USA |
| 40 | As18041 | GK Iringo | husked | HUN | 60 | As18061 | Maldwyn | husked | GBR |
| 41 | As18042 | Gregor | husked | CZE | 61 | As18062 | Marco Polo | naked | CZE |
| 42 | As18043 | Husky | husked | GER | 62 | As18063 | Maris Oberon | husked | GBR |
| 43 | As18044 | Hynek | naked | CZE | 63 | As18064 | Martin | husked | NOR |
| 44 | As18045 | APR166 | husked | UNKNOWN | 64 | As18065 | Mediteran | husked | SCG |
| 45 | As18046 | Ivore | husked | FRA | 65 | As18066 | Melys | husked | GBR |
| 46 | As18047 | Izak | naked | CZE | 66 | As18067 | Milton | husked | USA |
| 47 | As18048 | Jaak | husked | EST | 67 | As18068 | Mojacar | husked | CZE |
| 48 | As18049 | Jawor | husked | POL | 68 | As18069 | Myriane | husked | NLD |
| 49 | As18050 | Johanna | husked | BEL | 69 | As18070 | Navaro | naked | CAN |
| 50 | As18051 | Kalle | husked | EST | 70 | As18071 | Neklan | husked | CZE |
| 51 | As18052 | Kamil | naked | CZE | 71 | As18072 | Norbert | husked | CZE |
| 52 | As18053 | Kermit | husked | IRL | 72 | As18073 | Nordstern | husked | GER |
| 53 | As18054 | Kertag | husked | CZE | 73 | As18074 | OA 504-5 | naked | CAN |
| 54 | As18055 | Korok | husked | CZE | 74 | As18077 | Ogle | husked | USA |
| 55 | As18056 | Lennon | naked | GBR | 75 | As18078 | Oliver | naked | CZE |
| 56 | As18057 | Leo | husked | GER | 76 | As18079 | Otakar | naked | CZE |
| 57 | As18058 | Lidya | husked | RUS | 77 | As18080 | Otee | husked | USA |
| 58 | As18059 | Longchamp | husked | FRA | 78 | As18081 | Pan | husked | CZE |

**Table 7.** Continued

| No. | ECN | Name of variety | Type | Country of origin | No. | ECN | Name of variety | Type | Country of origin |
|---|---|---|---|---|---|---|---|---|---|
| 79 | As18060 | Maelor | husked | GBR | 99 | As18082 | Patrik | naked | CZE |
| 80 | As18083 | Pennlo | husked | USA | 100 | As18104 | Tibor | naked | CZE |
| 81 | As18084 | Polaris | husked | ARG | 101 | As18105 | Tjumenski golozernõi | naked | RUS |
| 82 | As18085 | Poncho | husked | FRA | 102 | As18106 | Valiant | husked | NLD |
| 83 | As18086 | Poseidon | husked | GER | 103 | As18107 | Veles | husked | CZE |
| 84 | As18087 | Pusahybrid | husked | IRL | 104 | As18108 | Veli | husked | FIN |
| 85 | As18088 | Radius | husked | CZE | 105 | As18109 | Vok | husked | CZE |
| 86 | As18089 | Ranch | black | FRA | 106 | As18110 | Walderm | husked | CAN |
| 87 | As18090 | Raven | black | CZE | 107 | As18111 | Yty | husked | FIN |
| 88 | As18091 | Rogar 8 | husked | ITA | 108 | As18113 | Zuton | naked | GBR |
| 89 | As18092 | Roope | husked | FIN | 109 | As18114 | Aarre | husked | FIN |
| 90 | As18093 | Rozmar | husked | CZE | 110 | As18115 | Canyon | husked | AUT |
| 91 | As18094 | Sagar | husked | CZE | 111 | As18117 | Efesos | husked | AUT |
| 92 | As18095 | Salo | husked | SWE | 112 | As18118 | Euro | husked | USA |
| 93 | As18096 | Salomon | naked | GER | 113 | As18119 | Jim | husked | FIN |
| 94 | As18097 | Sang | husked | SWE | 114 | As18120 | Katri | husked | CAN |
| 95 | As18098 | Santini | naked | CZE | 115 | As18121 | Riel | husked | CAN |
| 96 | As18099 | Saul | naked | CZE | 116 | As18122 | Scorpion | husked | GER |
| 97 | As18100 | Seldon | husked | CZE | 117 | As18123 | Celeste | black | CZE |
| 98 | As18101 | Shadow | naked | CAN | 118 | As18124 | SG-K 16370 | husked | CZE |

**Table 7.** Continued

| No. | ECN | Name of variety | Type | Country of origin | No. | ECN | Name of variety | Type | Country of origin |
|---|---|---|---|---|---|---|---|---|---|
| 119 | As18102 | Sirene | black | FRA | 136 | As18125 | SG-K 16564 | husked | CZE |
| 120 | As18126 | SG-K 16658 | husked | CZE | 137 | As18146 | Mansholts III | husked | NLD |
| 121 | As18127 | Merlin | husked | CZE | 138 | As18147 | Tucana | husked | AUS |
| 122 | As18128 | SG-K 16472 | husked | CZE | 139 | As18148 | Troshaver uit Besel | husked | NLD |
| 123 | As18129 | SG-K 16654 | husked | CZE | 140 | As18149 | Wodan | husked | NLD |
| 124 | As18130 | SG-K 16562 | husked | CZE | 141 | As18150 | Zandster | husked | NLD |
| 125 | As18131 | SG-K 6027 | naked | CZE | 142 | 03C0700057 | Buddah | husked | AUS |
| 126 | As18132 | Aragon | husked | CZE | 143 | 03C0700060 | Jongensklip | husked | ZAF |
| 127 | As18133 | Banquo | husked | GBR | 144 | 03C0700065 | Weston II | husked | IND |
| 128 | As18136 | KWS Contender | husked | GER | 145 | 03C0700088 | Rouge 31 | husked | DZA |
| 129 | As18137 | Matilda | husked | SWE | 146 | 03C0700089 | Precoce de Maroc | husked | MAR |
| 130 | As18138 | Max | husked | DNK | 147 | 03C0700130 | Klein 69 B | husked | ARG |
| 131 | As18140 | Astor | husked | NLD | 148 | 03C0700185 | Bundy | husked | AUS |
| 132 | As18141 | Auteuil | black | FRA | 149 | 03C0700287 | NP1 | husked | IND |
| 133 | As18142 | Gambo | husked | NLD | 150 | 03C0700288 | NP2 | naked | IND |
| 134 | As18144 | Kanota | husked | USA | 151 | 03C0700396 | La Prevision 7 | husked | ARG |
| 135 | As18143 | Prairie | husked | USA | | | | | |

ARG – Argentina, AUS – Australia, AUT – Austria, BEL – Belgium, CAN – Canada, CZE – Czech Republic, DNK – Denmark, DZA – Algeria, EST – Estonia, FIN – Finland, FRA – France, GBR – Great Britain, GER – Germany, HUN – Hungary, IND – India, IRL – Ireland, ITA – Italy, MAR – Morocco, NLD – Netherland, NOR – Norway, POL – Poland, RUS – Russia, SCG – Serbia and Montenegro, SWE – Sweden, USA – United States of America, ZAF – South Africa, ECN – national accession number

## 3.2. Extraction of Seed Storage Protein

For avenin extraction of single seed samples, four oat seeds from the set of samples of each variety were randomly selected, crushed separately with a hammer and mixed by vortexing (MS2 Minishaker, IKA, Germany) with 0.25 ml of a solvent consisting of 25% (v/v) 2-chlorethanol, 2% (w/v) SDS (sodium dodecyl sulfate) and 0.05% (w/v) pyronin Y (Sigma-Aldrich, USA). For bulk samples, twenty seeds were randomly selected and crushed in a grinding mill (Bosch MKM 6003, Slovenia). From the crushed seed mixture, four samples of 0.25 mg each were weighed and put into the Eppendorf tube and extracted by adding 0.25 ml of the same solvent that was used for single seed samples.

All samples were allowed to extract at room temperature for four hours. Afterward, they were centrifuged (Universal 32R Hettich Centrifugen, Germany) at 15000 rpm for ten minutes; the supernatant was then removed and put into new small tubes. This procedure was performed four times per each variety in case of single seed samples analysis and in duplicate for each variety in bulk sample analysis. All samples were stored in the refrigerator.

## 3.3. Avenin Separation by SDS-PAGE

Avenins were separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis), according to Laemmli (1970), with some minor changes. The polyacrylamide gel (180 x 160 x 0.75 mm) with 12% (w/v) resolving gel of pH 6.8 and 4% stacking gel of pH 8.8 was prepared according to the composition shown in Table 8.

**Table 8.** Chemical composition of the gel used for the protein separation

| **Resolving gel 12%** | **Stacking gel 4%** |
|---|---|
| 29 ml Acrylamide and Bis solution (Bio-Rad, Germany) | 4 ml Acrylamide and Bis solution (Bio-Rad, Germany) |
| 63 ml Tris 8.8 (Sigma-Aldrich, USA) | 13.4 ml Tris 6.8 (Sigma-Aldrich, USA) |
| 0.72 ml 10% SDS (Sigma-Aldrich, USA) | 0.26 ml 10% SDS (Sigma-Aldrich, USA) |
| 7 ml Distilled water | 8.8 ml Distilled water |
| 0.72 ml 10% Ammonium persulfate | 0.26 ml 10% Ammonium persulfate |
| 42.6 µl TEMED (Sigma-Aldrich, USA) | 10.6 µl TEMED (Sigma-Aldrich, USA) |

Each well on the gel was loaded with 0.15 µl of extract, four wells per one variety in case of single seed samples, and two wells per one variety in case of bulk samples. Two commercially purchased protein ladders – SM (SigmaMarker wide range 6,500 – 2,000,000 Da, Merck, Czech Republic) and TS (Thermo Scientific™ PageRuler™ Unstained Broad Range Protein Ladder, Thermo Fischer Scientific Life Scientific, Czech Republic) were used as molecular weight standard markers. Both standard markers were used in the amount of 0.1 µl per one well. Moreover, two internal markers were created. According to the broad protein band profile, two varieties Poncho and Ranch were chosen as they were evaluated as the most polymorphic (Figure 5). Both markers were used in the amount of 0.15 µl of extract per one well.

After filling the wells and transferring the gel sandwich into vertical electrophoresis Hoefer SE 600 (Hoefer, USA), the buffer composed of 0.25 M Tris and 1.92 M glycine (8.3 pH) (Bio-Rad, Germany) was poured into buffer chamber. The electrophoretic system was set on 50 mA and after 30 minutes raised on 60 mA and then let run for about three hours.

After the end of electrophoretic separation, the gels were fixed for 30 minutes in 20% (w/v) solution of trichloroacetic acid and stained for 24 hours with a solution of 0.05% (w/v) Coomassie Brilliant Blue (CBB) R250 (SERVA, Germany), 25% (w/v) methanol (Lach-ner, Czech Republic) and 10% (w/v) acetic acid (Lach-ner, Czech Republic). Later, gels were bleached in a mixed solution of 25% (w/v) methanol and 10% (w/v) acetic acid for 1 hour. Afterward, gels were immersed in distilled water for several days to remove the remaining color and scanned into the computer.

## 3.4. Gel Evaluation and Statistical Analysis

For evaluation of band positions on the gel, internal markers had to be created. Ten oat varieties were chosen from the list of all samples based on the available scientific literature and their distinct celiac reactivity. In the chosen sample set, the celiac reactivity was very various (Ballabio et al. 2010; Mujico et al. 2011), so it was expected that also the avenin band spectra would be various. Potential internal markers were analyzed by SDS-PAGE and compared with each other (Figure 5). The analysis was first done on 10% gel, but during the development of the correct methodology that achieves the best results, it was decided to use 12% gel, as it is described in Table 8. The two most different varieties were searched to ensure covering as broad protein band spectrum as possible. As a result, two French varieties Poncho and Ranch, were selected to become the internal markers.

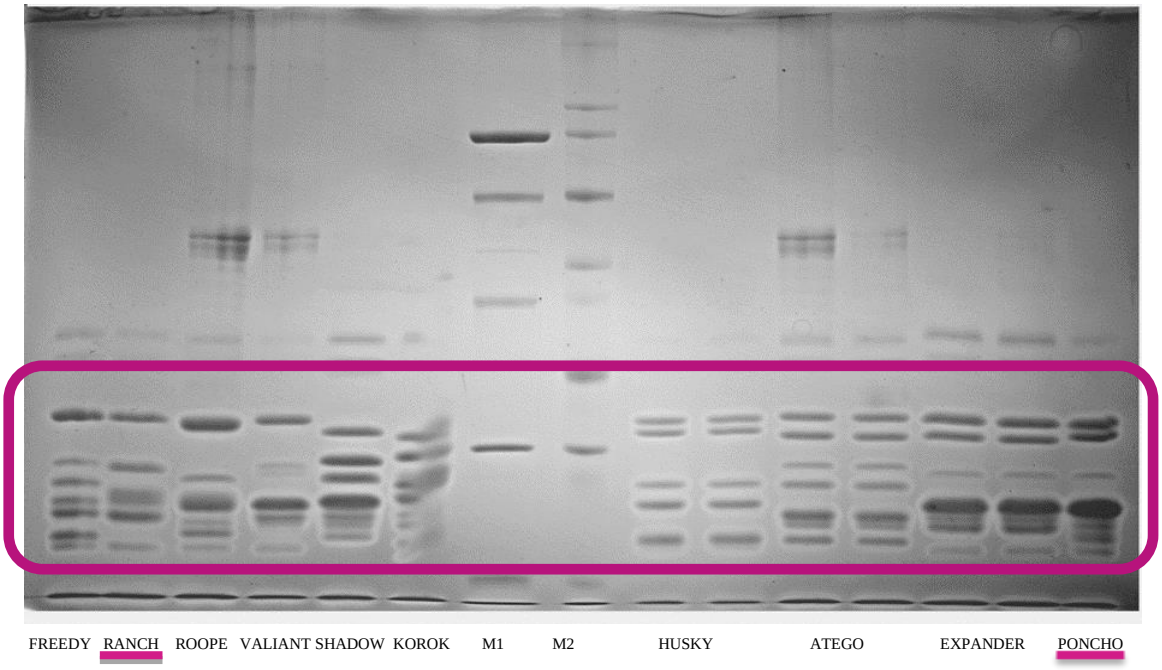


**Figure 5.** Ten chosen varieties analyzed by SDS-PAGE on 10% gel with bands in the molecular weight range of 18 – 35 kDa. M1 is a Low Range BioRad marker; M2 is a Wide Range Sigma marker. Internal markers Poncho and Ranch were marked with a purple underline

Avenin bands were analyzed in the molecular weight range of 18 – 35 kDa. Bands of internal markers and analyzed samples located in this range were expressed by numbers ranging from $1^+$ to 12* ($1^+$, 1, 1*, 2, 2*, 3, 3*, 4, 4*, 5, 5*, 6, 6*, 7, 7*, 8, 8*, 9, 9*, 10, 10*, $11^+$, 11, 11*, 12, 12*). The symbols * and + indicates the middle position of the band. For example, symbol * labels position, that is between two other positions marked by numbers without symbol (e.g., position 11* is slightly under position 11 but does not reach the same position as band 12, that is under them). The symbol + highlights position that is slightly above the band of the same number (e.g., position $11^+$ is above band 11, but under 10*) (Figure 6).

The positions of protein bands were then evaluated as presence (1) or absence (0) of a given band. From this data, a binary matrix was prepared. The genetic relationship was evaluated by calculating Jaccard´s dissimilarity coefficient using Darwin 5 software version 5.0.158, and then the UnWeighted Neighbor-Joining method was used for the construction of a final Neighbour Joining (NJ) dendrogram (Saitou & Nei 1987).

# 4. Results

A total of 151 oat varieties were analyzed using the SDS-PAGE method. From the total, 117 analyzed samples originated in Europe (covering 17 European countries), 16 samples originated in North America. Eight samples had Asian origin; three samples originated in Africa, South America, and Australia. One sample had an unknown origin.

## 4.1. Internal Markers and Gel Evaluation

The SDS-PAGE spectra of all examined varieties were analyzed predominantly in molecular weight from 18 to 35 kDa. Altogether, 26 different band positions were expressed by numbers ranging from $1^{+}$ to 12* (see chapter 3.4. Gel Evaluation and Statistical Analysis). All bands are marked on the gel below (Figure 6).

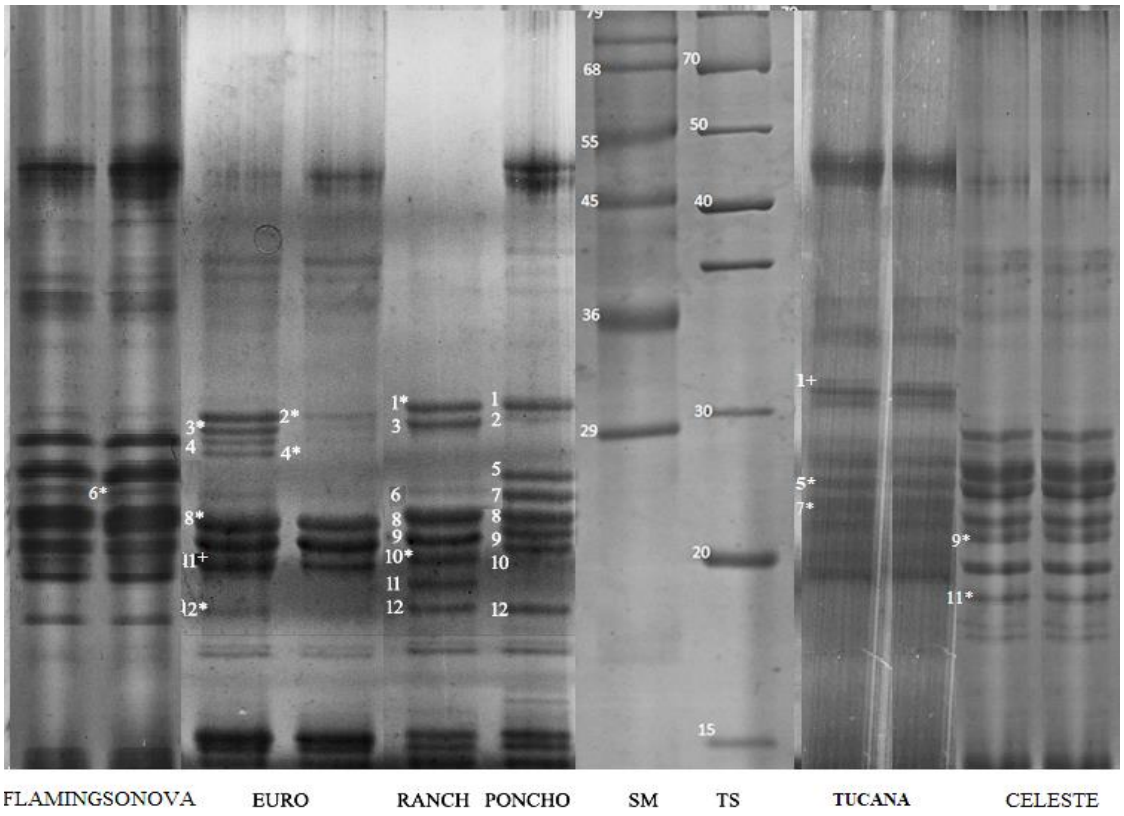


**Figure 6.** SDS-PAGE spectra of four bulk samples (varieties Flamingsnova, Euro, Tucana, Celeste) and two internal markers (Ranch, Poncho) with numbered bands. Commercial markers SM (SigmaMarker wide range 6,500 – 2,000,000 Da) and TS (PageRuler™ Unstained Broad Range Protein Ladder) shows the molecular weight range (kDa). Avenins were evaluated only in molecular weight of 18 – 35 kDa

## 4.2. Avenin Analysis of the Single Seed Samples

The avenin analysis by the single seed method was performed on 127 varieties. For each variety, four seeds were randomly chosen from the set and analyzed individually. Generally speaking, there is variability among all samples because no identical band composition was observed in the whole collection. However, 29% of all analyzed varieties were also found to have intra-varietal polymorphism, meaning that samples were variable in band positions among analyzed individual seeds of a given variety. For confirmation or disprove of intra-varietal variability, the samples were examined at least twice. The inconsistencies were manifested by lack or excess of one or several bands.

The rest of the samples (71%) were evaluated as non-variable in avenin band pattern. The number of bands of non-variable samples was oscillating between 6 – 10 bands per sample, reaching extreme values in only several cases. Only two varieties reached the number of 10 bands – Cavaliere (black variety, Czech Republic) and Flamingsnova (husked variety, Germany) (Figure 7).

The frequency of individual bands (alleles) was ranging between 0% to nearly 68%. The highest frequency of occurrence across the samples was observed in band 8 (23.5 kDa), followed by band 9 (22.0 kDa), with nearly 67%. On the other hand, protein bands of numbers $1^{+}$ (1%), 9* (3%), and 12* (6%) were almost absent. Bands with number 2* were absent completely.

In the case of the variable seeds, the total amount of bands was also mostly oscillating between 5 to 10 bands per sample. For example, husked variety Dalimil originating in the Czech Republic had six bands in one sample, but three remaining samples showed five bands for each, meaning that one band was missing. The example of the high number of avenin protein bands was Classic – husked variety coming from the USA, displayed significant intra-varietal polymorphism because only two analyzed seeds had the same number of bands. One from the two remaining samples had ten bands, and one sample had 11 bands (Figure 7). The allele frequency values were in the range of 1 – 75%. The highest frequency was reached by band 8, whereas the lowest frequency occurred by 2* (1%), 9* (4%), and 12* (4%).

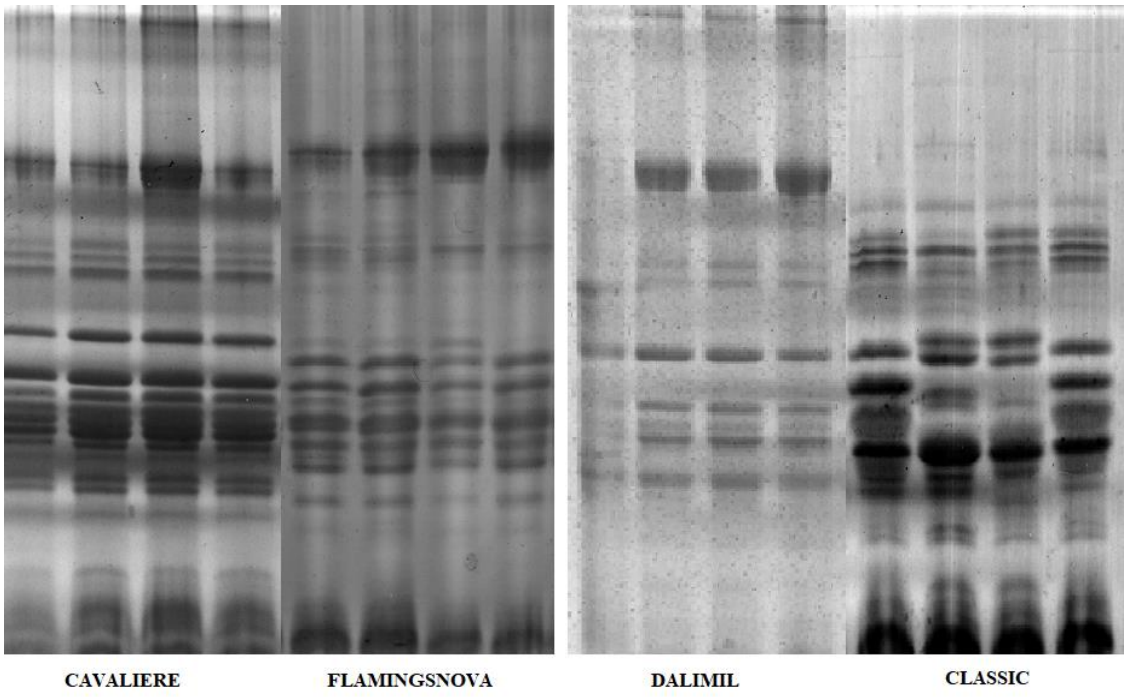


**Figure 7.** Varieties Cavaliere and Flamingsnova with the highest number of protein bands of all analyzed single seed samples. Varieties Dalimil and Classic show intra-varietal polymorphism

To compare variable and non-variable samples, the similarities, as well as differences, occurred. The band number range was almost the same, with several exceptions in the frequency of some alleles. In both cases, allele no. 8 was the most common, whereas bands 2*, 9*, and 12* were absent or appeared in low frequencies.

The main difference could be seen in band 1+, which is nearly absent in the case of non-variable seeds; however, it reaches a frequency of 8% in variable samples. From a total of 26 band positions, nine avenin band positions were reached with similar allele frequencies (less than 4% difference). On the other hand, the most significant dissimilarity was observed in allele 10, with a 20% difference in frequencies of variable and non-variable samples (Table 9).

**Table 9.** Comparison of allele frequencies of variable and non-variable single seed samples in avenin spectra

| Alleles | Alleles frequency of variable seed samples (%) | Alleles frequency of non-variable seed samples (%) | kDa |
|---|---|---|---|
| $1^{+}$ | 8 | 1 | 30.5 |
| 1 | 39 | 39 | 30.2 |
| 1* | 32 | 39 | 30.0 |
| 2 | 20 | 12 | 29.5 |
| 2* | 1 | 0 | 29.7 |
| 3 | 23 | 31 | 29.0 |
| 3* | 20 | 23 | 28.5 |
| 4 | 20 | 33 | 28.0 |
| 4* | 7 | 10 | 27.5 |
| 5 | 38 | 36 | 26.5 |
| 5* | 19 | 9 | 26.0 |
| 6 | 32 | 42 | 25.5 |
| 6* | 11 | 18 | 25.0 |
| 7 | 50 | 37 | 24.5 |
| 7* | 9 | 23 | 24.0 |
| 8 | 76 | 68 | 23.5 |
| 8* | 10 | 17 | 23.0 |
| 9 | 51 | 67 | 22.0 |
| 9* | 4 | 3 | 21.0 |
| 10 | 51 | 31 | 20.5 |
| 10* | 49 | 49 | 20.0 |
| $11^{+}$ | 13 | 13 | 19.8 |
| 11 | 43 | 56 | 19.2 |
| 11* | 32 | 23 | 19.0 |
| 12 | 34 | 44 | 18.8 |
| 12* | 2 | 6 | 18.5 |

## 4.3. Avenin Analysis of the Bulk Samples

A total of 151 oat varieties were analyzed for the avenin band spectrum as bulk samples. For each variety, the gel visualization was performed in duplicate to ensure their similarity. According to the obtained results, all analyzed samples were variable with a lack of the same band composition among varieties. The total number of bands ranged from 5 – 11 bands; however, the highest value was reached only in two cases in varieties Sirene and CC4146 (Figure 8). Both were black oat varieties of different geographical origins – Sirene originated in France and CC4146 in Great Britain.

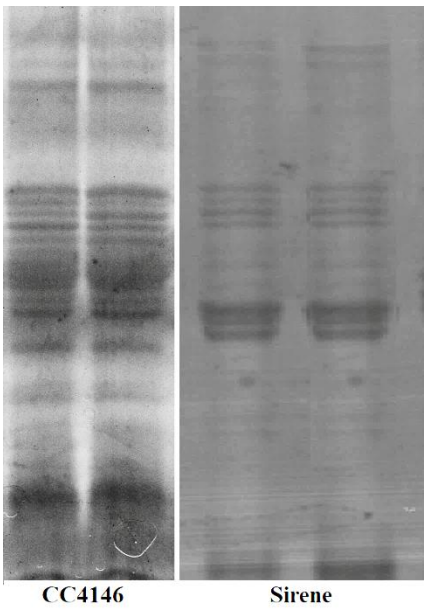


**Figure 8.** Varieties CC4146 and Sirene with the highest amount of protein bands

The frequency of the individual bands was ranging between 1% to 64%. The most frequently occurring bands were marked as no. 8 (23.5 kDa), showing up in nearly 64% of all studied samples, and no. 9 (22.0 kDa) occurred in 56% of cases. The least frequent bands were noticed in positions $1^{+}$ (1%), 2* (3%) and 9* (5%) (Table 10).

Bulk samples were also analyzed by calculation using software Darwin 5. The resulting dendrogram divided the samples into six more or less related clusters according to obtained avenin band spectra. The dendrogram also covered the geographical origin of the samples, marked by different colors (Figure 9).

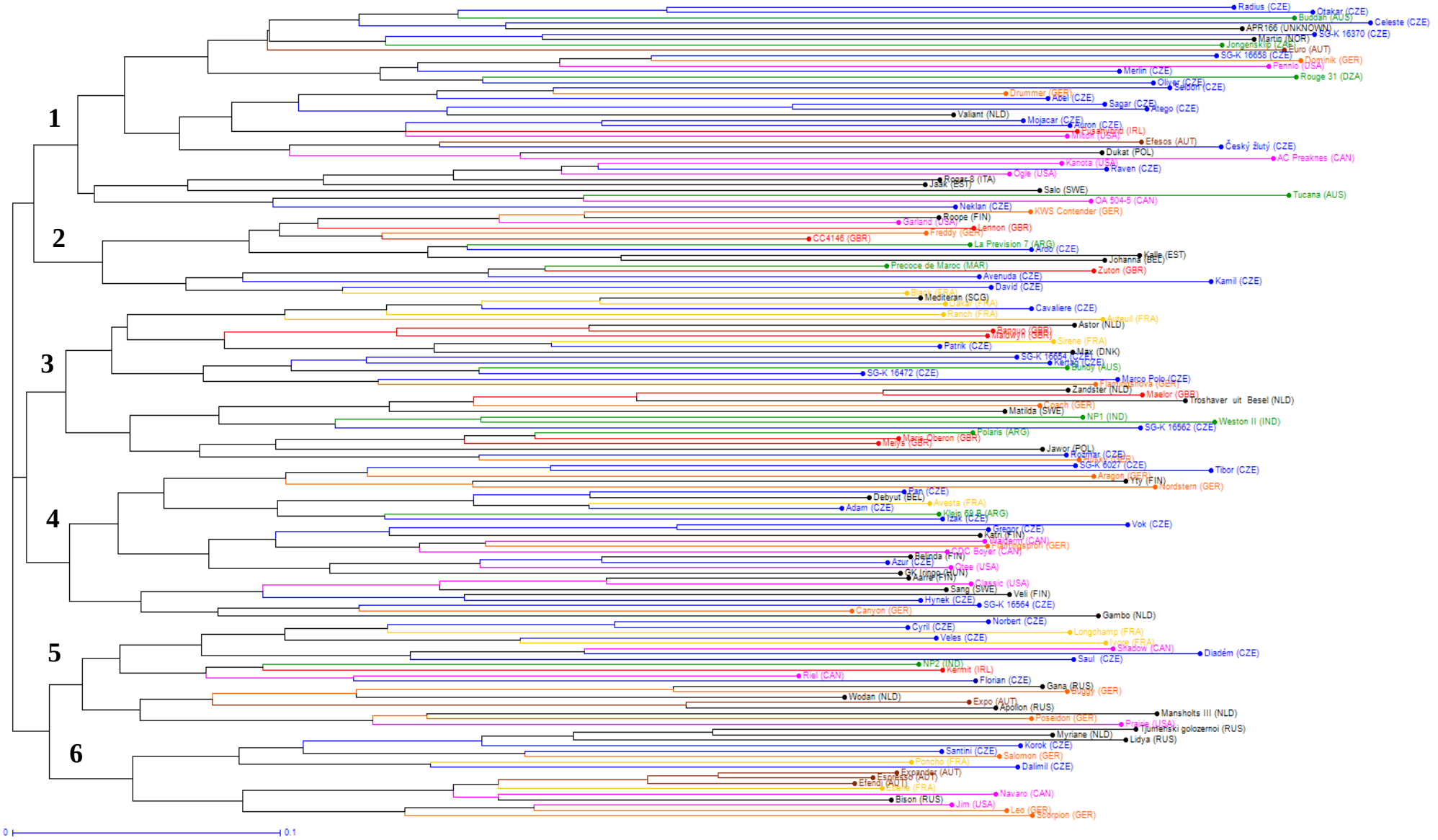


**Figure 9.** Dendrogram created according to avenin protein band spectra of 151 bulk samples. Origin of the varieties is included and marked by colours. **Blue –** Czech varieties, **orange –** German varieties, **red –** Great Britain and Ireland varieties, **yellow –** French varieties, **brown** – Austrian varieties, **pink –** North American varieties, **green –** remaining non-European countries (Argentina, Australia, India, Morocco, South Africa), **black –** remaining European countries (Belgium, Denmark, Estonia, Finland, Hungary, Italy, Netherland, Norway, Poland, Serbia and Montenegro, Sweden) and Russia

Individual clusters were analyzed separately. Cluster 1 covered 38 varieties altogether. In total, 29 varieties had European origin, from which 16 originated in the Czech Republic. There was also the highest presence of North American varieties, and one-third of all tropical/subtropical varieties were presented in this cluster. In nearly 65% of analyzed cases, there was a match in the band no. 7*. Comparing the other clusters, this band seems to be unique for cluster 1 because its occurrence in other clusters is quite rare (e.g., in cluster 5 reached 40% of similarity, but in clusters 6, 4, and 2, it was nearly or completely absent).

This cluster also had a high concentration of several bands that reached very low allele frequencies (Table 10) in the whole set of samples. Those are bands no. 1+, 4*, 5*, 6* and 12*. Generally, this cluster could be rated as the least consistent in terms of the band position similarity as the match percentage reached the lowest values.

Cluster 2 (Figure 11) included 16 varieties. The majority of the varieties (13) were bred in Europe; however, in terms of specific varieties´ origin, cluster 2 is the most diverse one. The presence of the tropical/subtropical varieties was lower compared to cluster 1 since there were only two varieties appearing in the cluster 2. Despite the wide geographical origin of included varieties, the similarity in avenin bands occurred in 81% in three different cases – in bands 5, 7, and 10, which is, compared to other clusters, relatively high rate. The analogy was also found in bands 1* and 8 that were similar for all 16 varieties for almost 69%.

In the third cluster, 31 varieties were presented. Four of them had a non-European origin; the rest originated in Europe. Most of the French and British varieties were represented in this cluster, as well as the second third of the tropical/subtropical varieties. In 81% of the cases, there was the conformity for bands 2 and 12. Three more bands matched in more than 50% of cases were bands numbered as 5, 8, and 10*.

In the case of cluster 4, most of the analyzed varieties had Czech origin. In addition, five out of six Finnish varieties were found exclusively in this cluster. The bands no. 6 and 8 coincided in 79% of the cases; however, the match occurred also in bands 1, 3, 10*, and 11, ranging between 50 – 70%.

Cluster 5 was quite diverse in terms of the origin of analyzed varieties, as well as cluster 2. Altogether, there were 11 different places of origin; however, more than half of the samples originated in Europe. Despite the broad origin, the similarity in the bands was very high, reaching 95% of conformity for band 9 and 90% for band 1*.

The highest similarity rate was reached in cluster 6 (Figure 12), where there was 100% conformity in band number 8. High conformity was also observed for bands 12 (94%), 1* (80%), 6, and 10* (71% each). The divergence of this cluster, compared to other clusters, was also given by the high presence of band 2*, that did not occur in any other cluster except cluster 2, where it appeared only one case. All included varieties originated in 8 different countries, with a high presence of Russian and Austrian varieties, compared to the other clusters.

According to the dendrogram results, the two most distant varieties were Radius from the Czech Republic and Scorpion from Germany. Although they are considered dissimilar, there are two bands on position 9 and 10*, that share the same position (Figure 10).

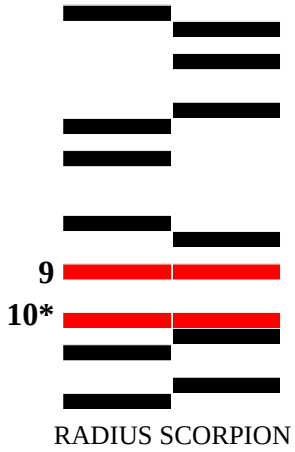


**Figure 10.** A schematic diagram with the protein band spectra of the two most distant varieties Radius and Scorpion. Bands 9 and 10* (red color) were shared in both varieties.

The results also confirmed the right choice of internal markers Poncho and Ranch, as they can also be considered distant. Poncho could be found in cluster 6, whereas Ranch was located in cluster 2. They both have only two similar bands on position 8 and 12.

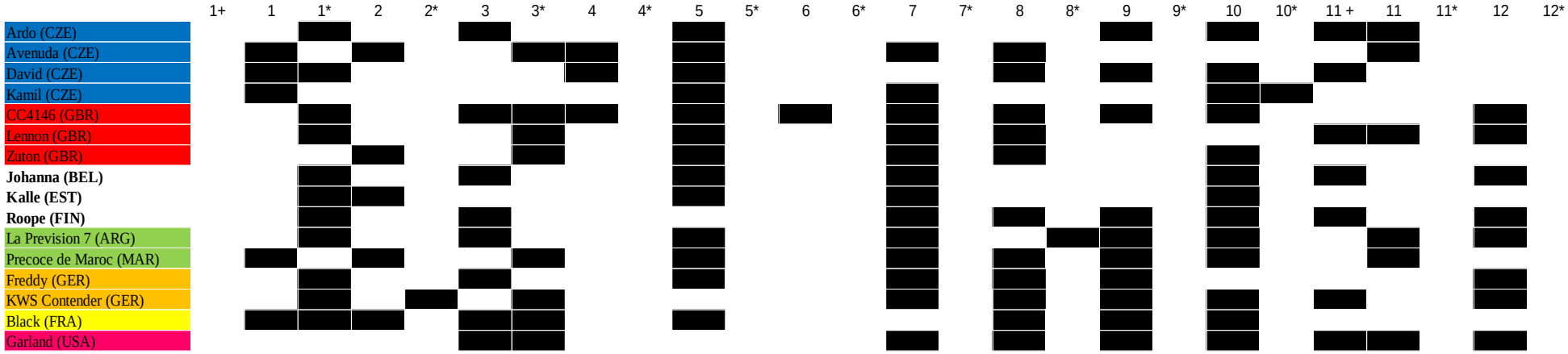


**Figure 11.** Schematic diagram of the cluster 2, demonstration high varietal origin diversity, with marked presence (black) or absence (white) of avenin bands and origin of the varieties *

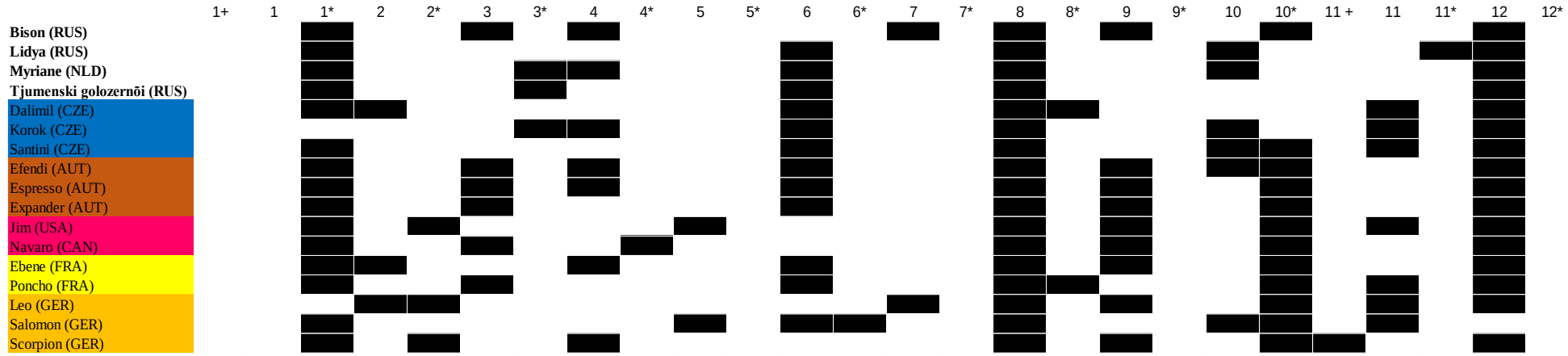


**Figure 12.** Schematic diagram of the cluster 6, showing the highest similarity rate among individual bands, with marked presence (black) or absence (white) of avenin bands and origin of the varieties *

* **Blue –** Czech varieties, **orange –** German varieties, **red –** Great Britain and Ireland varieties, **yellow –** French varieties, **brown –** Austrian varieties, **pink –** North American varieties, **green –** remaining non-European countries (Argentina, Australia, India, Morocco, South Africa), **black –** remaining European varieties (Belgium, Estonia, Finland, Netherland) and Russian varieties.

### 4.3.1. Origin of the Samples and Avenin Variability

The dendrogram results (Figure 9) also demonstrated avenin variability according to the geographical origin of the variety. The varieties originating in Austria (Figure 13) and France appeared to be the most consistent because they show a relatively similar pattern of the avenin band positions, therefore lower avenin polymorphism. Austrian varieties were also located closer to each other in the dendrogram, mainly in cluster 6 (Figure 9). Almost entirely consistent on avenin level were varieties originating in Finland since five out of six analyzed Finnish samples clustered relatively close to each other. Similar results were observed for samples originating in the UK and Ireland. Both groups of samples with Finnish and the UK/Ireland origin reached the similarity rate equal to or higher than 50% in total of six different avenin bands.

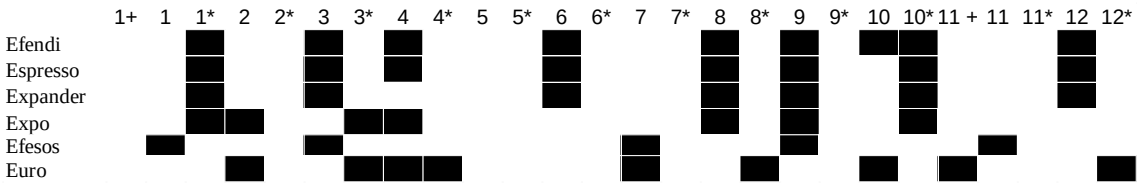


**Figure 13.** Schematic diagram of Austrian varieties showing similar avenin band pattern

A slightly higher level of avenin polymorphism was observed in USA/Canadian, German, Russian, and Netherland varieties with similarity equal or higher than 50% in 5 different bands for each group. The particular group that included samples from northern countries (Estonia, Norway, Sweden, and Denmark) was analyzed with a result of a higher degree of avenin polymorphism since the similarity was reached in 4 bands only; however, after including the Finnish varieties in this group, the avenin polymorphism slightly decreased.

Varieties originated in non-European and non-American countries (marked as green in dendrogram) were generally labeled as subtropical or tropical. Altogether, 12 varieties were belonging to this group. The origin of those varieties was diverse (Argentina, Algeria, Australia, India, Morocco, South Africa); however their avenin

polymorphism rate reached only slightly higher values. Namely, five bands no. 3, 5, 9, 11+, and 11 demonstrated the similarity rate equal to or higher than 50%.

By comparing those varieties separately, based on their geographical origin, the results showed relatively high consistency in samples originating in Argentina, India and on the African Continent (South Africa, Morocco, Algeria), because each of the groups had seven bands with similarity rate equal to or higher than 50%. Although Australian varieties contained only five protein bands, that had similar positions in 50% or more, varieties Tucana and Buddah were more similar to each other than Bundy since they shared completely the same protein bands in four cases. Similarly, the Indian varieties Weston II and NP1 had more similar band patterns than NP2, because they were sharing six out of nine found protein bands.

Tropical/subtropical varieties appeared mainly in clusters 1 and 3, except varieties Klein 69B (Argentina), La Prevision 7 (Argentina), Precoce de Maroc (Morocco), and NP2 (India) that were separated in other clusters. According to data obtained from dendrogram layout, those varieties were related to European varieties, most often to the Czech varieties (in six cases), then British/Irish varieties (in three cases) and Canadian or Norway varieties (both in one case each) as their avenin band pattern was approximately similar.

The avenin band layout of subtropical/tropical variety and its closes European or Canadian varieties (located always below the given tropical/subtropical variety) is given in Figure 12 for cluster 1 and in Figure 13 for cluster three. Figure 14 was created for remaining varieties that were not clustering with the other included varieties.

The highest polymorphism in band spectra was observed among the samples originating in the Czech Republic, which was also the most numerous group of samples. The avenin band similarity equal or higher than 50% occurred in only three cases, in bands no. 9, 10* and 11. The distribution of Czech samples was relatively similar across the whole dendrogram spectra; however, their highest quantity was visible in cluster one and three.

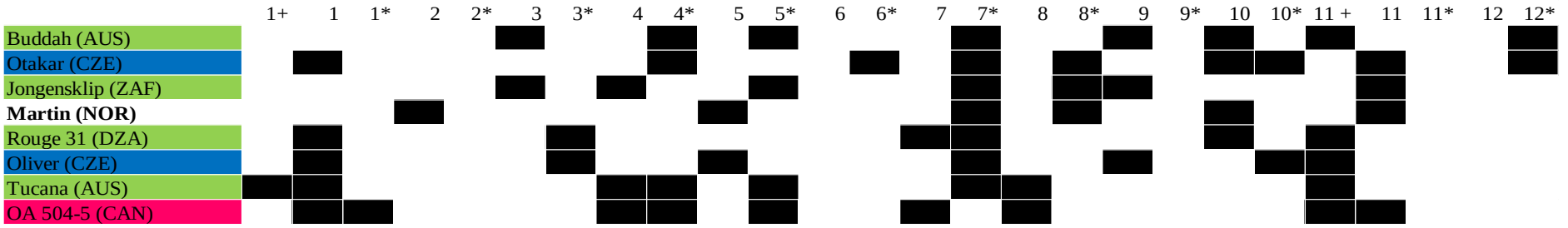


**Figure 14.** Schematic diagram of tropical/subtropical varieties and their related varieties from cluster 1 with marked presence (black) or absence (white) of avenin bands and origin of the varieties *

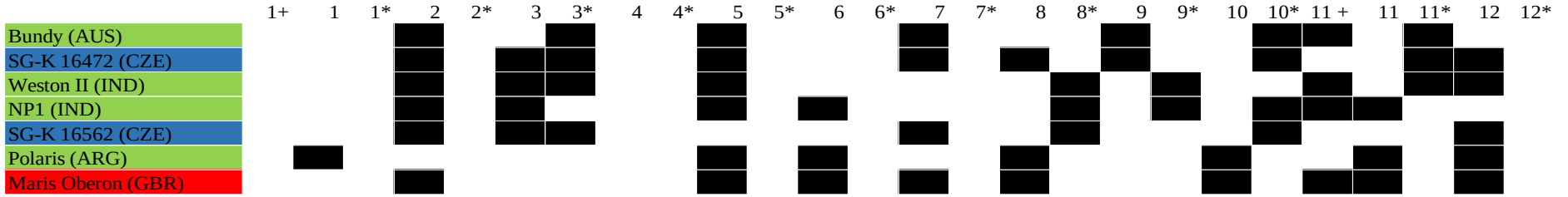


**Figure 15.** Schematic diagram of tropical/subtropical varieties and their related varieties from cluster 3 with marked presence (black) or absence (white) of avenin bands and origin of the varieties *

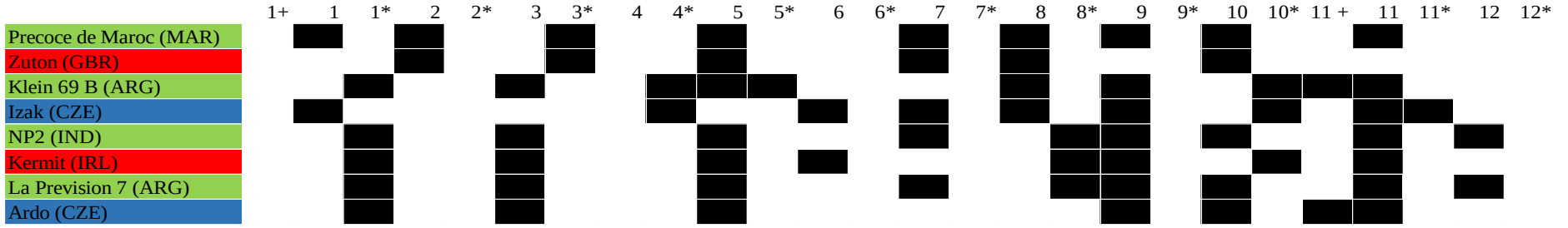


**Figure 16.** Schematic diagram of remaining tropical/subtropical varieties and their related varieties with marked presence (black) or absence (white) of avenin bands and origin of the varieties *

* **Blue** – Czech varieties, **red –** Great Britain and Irish varieties, **pink** – Canadian variety, **green** – remaining non-European countries (Argentina, Australia, Algeria, India, Morocco, South Africa), **black –** remaining European countries (Norway)

## 4.4. Comparison in Avenin Spectra of the Single Seed and Bulk Samples

The results showed that there are several differences but also similarities between single seed and bulk samples. The number of bands was more or less similar in both types of avenin analysis, as well as the band with the highest frequency (band no. 8) and the lowest frequency bands (numbers 1+, 2* and 9*) (Table 10).

The specific region with the presence of celiac reactive peptides is around 25 – 37 kDa region (Comino et al. 2016). This molecular weight corresponds to the positions identified among bands 1+ to 6* (30.5 to 25 kDa). Alleles frequencies in this region were generally reaching lower numbers for single seeds than for bulk analysis. The exceptions are only in bands 1+ and 1.

The highest alleles frequency difference was observed in band 2 (26% difference) and 3 (14% difference), whereas band 1+, 4, 5, 5*, and 6 had almost similar frequencies of avenin alleles between individual and bulk samples. Bands 2, 3, 3* and 4 reached frequencies lower than 30% in case of single seed analysis, but higher than 30% in bulk seed analysis. The remaining bands (1+, 2*, 4*, 6*) reached negligible frequencies (less than 15%) in both cases (Table 10).

In samples analyzed by bulk method, several varieties contained a maximum of one band in the reactive protein spectrum (25 – 37 kDa) – Maelor (Great Britain), Shadow (Canada), and Troshaver uit Besel (Netherland) and 24 different varieties containing a maximum of two bands in reactive protein band spectrum. In the case of single seed analysis, there were also three varieties with only one avenin band in reactive protein spectra – Maelor (Great Britain) Polaris (Argentina), Rozmar (Czech Republic).

On the other hand, the highest number of bands in the reactive spectrum for bulk samples was observed by varieties Black, CC4146, Sang, and Sirene. All those varieties had six protein bands in the reactive spectrum. For the single seed analysis, the number of bands in this spectrum was very various, and it generally oscillated between two to four bands.

The region between bands no. 7 to 12* (26.5 – 18.5 kDa) was also variable in the band frequency range, but less than the first region. The highest difference in alleles frequency was reached in case of band 11+ (15% difference), whereas the remaining

bands reached a difference of 9% or less. Nine out of 17 samples had nearly similar allele frequency (3% difference or less). Bands no. 7*, 8*, 9*, 11+, 11*, and 12* reached frequency lower than 28% in both types of avenin analysis. On the contrary, the remaining bands gained allele frequencies in the range of 32 – 70%.

**Table 10.** Comparison of allele frequencies of avenin spectra obtained through individual and bulk samples analysis

| Alleles | Alleles frequency of individual seed samples (%) | Alleles frequency of bulk samples (%) | kDa |
|---|---|---|---|
| $1^{+}$ | 2 | 1 | 30.5 |
| 1 | 39 | 30 | 30.2 |
| 1* | 37 | 39 | 30.0 |
| 2 | 14 | 40 | 29.5 |
| 2* | 0 | 3 | 29.7 |
| 3 | 29 | 43 | 29.0 |
| 3* | 22 | 30 | 28.5 |
| 4 | 29 | 32 | 28.0 |
| 4* | 9 | 15 | 27.5 |
| 5 | 37 | 40 | 26.5 |
| 5* | 12 | 9 | 26.0 |
| 6 | 40 | 46 | 25.5 |
| 6* | 16 | 5 | 25.0 |
| 7 | 41 | 39 | 24.5 |
| 7* | 19 | 28 | 24.0 |
| 8 | 70 | 64 | 23.5 |
| 8* | 15 | 13 | 23.0 |
| 9 | 62 | 56 | 22.0 |
| 9* | 4 | 5 | 21.0 |
| 10 | 37 | 32 | 20.5 |
| 10* | 49 | 46 | 20.0 |
| $11^{+}$ | 13 | 28 | 19.8 |
| 11 | 52 | 51 | 19.2 |
| 11* | 26 | 17 | 19.0 |
| 12 | 42 | 41 | 18.8 |
| 12* | 4 | 6 | 18.5 |

# 5. Discussion

## 5.1. Molecular Weights and Avenin Bands Spectra

The SDS-PAGE analysis of avenin band spectra detected 26 different allelic positions in the molecular weight range of 18 to 35 kDa; however, in some studies, the authors define a much more extensive range that covers resulting bands as avenin protein bands. Dumlupinar et al. (2011) reported avenin protein bands ranging from 8.8 to 45 kDa by analysis of 196 Turkish oat landraces, similarly to Gregorova et al. (2015), that reported avenin molecular weight range from 8 to 45 kDa of 12 varieties with various origin (Slovakia, Germany, Czech Republic). Peterson´s et al. (1987) results showed a molecular weight of avenins between 22 to 43 kDa after analysis of a Froker variety. On the other hand, Mickowska et al. (2015) reported a very narrow avenin molecular weight range from 25 to 35 kDa by analysis of different oat products from Finland and Poland.

The most similar results compared to the findings in this thesis were obtained by several studies such as Dvořáček et al. (2003) with the avenin band spectrum ranging between 20 to 36 kDa, which was observed after analysis of three Czech and two German cultivars. Benoit et al. (2017) analyzed 20 different oat samples and reported the avenin molecular weight range of 14 – 35 kDa.

A total amount of different allelic positions was confirmed by the study of Portyanko et al. (1998), where there were 26 avenin alleles discovered in a total of 252 oat varieties of various origin; however, variations in the number of bands per sample were indicated. In this study, avenin analysis of the single seed samples showed that the number of bands oscillated between 6 – 10 and between 5 – 11 in bulk samples analysis. Compared to the other studies, Dumlupinar et al. (2011) observed 4 to 16 different protein positions, while Jussila et al. (1992) analyzed 7 to 14 avenin bands in 28 Finnish varieties and Portyanko et al. (1998) described similarly 7 to 11 avenin bands.

The fact that the results of molecular weights and band quantity diverge might be caused by the diversity of the varieties that were subjected to examination. Samples analyzed in this study originated in 20 different countries, but several of the discussed papers generally examined varieties of the same origin (Peterson et al. 1987; Jussila et

al. 1992; Dumlupinar et al. 2011). The selected method of avenin analysis can also play an important role. Two of the discussed studies used A-PAGE (Portyanko et al. 1998, Gregorova et al. 2015) instead of SDS-PAGE.

The main difference between these two methods is that A-PAGE uses the low pH environment to make proteins positively charged and separates them according to the size. In this system, the number of detected bands is usually higher than in SDS-PAGE (Gordon 1969). The SDS-PAGE uses dodecyl sulfate to charge the proteins negatively and separates them according to their molecular weight. It is possible to use the molecular weight marker in this system (Deyl 1979). Unlike A-PAGE, SDS-PAGE cannot perform separation of proteins of similar size but different charges (Gordon 1969). The methodology of mentioned researches also differs since some varieties were examined as single seed samples (Jussila 1992; Portyanko 1998; Dvořáček et al. 2003), while others were analyzed as the bulk samples (Gregorova et al. 2015; Mickowska et al. 2015; Dumlupinar et al. 2011).

## 5.2. Degree of Polymorphism in Avenin Band Spectra

In general, the polymorphism of avenins is heterogeneous and high enough, as it could be used in the oat variety identification (Hansen et al. 1988; Gregorova et al. 1996; Dvořáček et al. 2003). This claim was confirmed by obtained results in this thesis, as there was not any identical avenin band pattern found. Moreover, this method is less demanding for equipment, more cost-effective and accessible, comparing to DNA-based methods (Polišenská et al. 2011).

The high degree of diversity in oat cultivars might be beneficial for breeding objectives by improving some of the valuable traits (Lookhart 1985; Achleitner et al. 2008). As reported by Ahokas et al. (2005), the potential of protein pattern polymorphism lies in the possibility of lowering the number of proteins that are harmful to CD patients.

However, the single seed avenin analysis showed that 29% of varieties had intra-varietal polymorphism, which is in contradiction with previously mentioned findings because it makes complicated or even impossible to identify individual varieties among each other. On the other hand, it is not excluded that intra-varietal polymorphism was not caused by contamination from other genotypes or by outcrossing during the

breeding process (Portyanko et al. 1998). Therefore, the use of bulk samples method is recommended, since there was no intra-varietal polymorphism observed.

Moreover, bulk method analysis of protein bands is producing stable results, that can easily distinguish cultivars among each other (Gardiner & Forde 1992), despite their cross-fertilization and high variability (Gardiner & Forde 1987, 1988). Although oats are generally considered as self-pollinators, the crosses between plants of the same variety (Grippe & Hayes 1925) occur depending on the place of growing and climatic conditions (Bickelmann & Leist 1985).

## 5.3. Varietal Relationships Based on the Dendrogram Results

The dendrogram results divided oat varieties examined by bulk avenin analysis into six main clusters. Although some varieties had a closer relationship based on their avenin band spectra, there was no repetition in the avenin band pattern, meaning that each oat variety was unique. The two most distant varieties – Radius (Czech Republic, breeding company Selgen Krukanice a.s.) and Scorpion (Germany, breeding company Saaten Union) – surprisingly shared two equal band position; however, it was assumed that such a distance would result in a complete difference without sharing any bands.

Another interesting fact is that considering the geographical origin, both varieties come from relatively close, neighboring countries. Historically, mostly German varieties were used in the breeding of Czech varieties (Petr & Húska et al. 1997); thus, the most distant varieties were expected to come from more distant countries. Based on the pedigree of variety Radius (Figure 15), two German varieties were used in the third generation; however, the pedigree of Scorpion was not possible to found, so proper genetic comparison cannot be demonstrated.

Austrian and French varieties appeared to be the most consistent in the avenin band spectrum, which was also confirmed by their clustering nearby each other in the dendrogram. The breeding influenced the similarity of Austrian varieties since they were bred in the same breeding program from Flamingsnova cross (Achleitner et al. 2008). The relatively steady pattern was demonstrated by Finnish varieties, which was previously confirmed by Jussila et al. (1992).

British and Irish varieties were also relatively uniform, but in all mentioned cases, it should be taken into account that the individual groups of samples with the same geographical origin were relatively small (less than ten).

The geographical origin of variety can be a sign of a closer relationship on the avenin level as well as on genetic level because there is a presumption that these varieties can have the same ancestors, that were used during the breeding process (Lookhart 1985; Portyanko et al. 1998). The correlation of the origin and similarity in the avenin pattern was also confirmed by several studies, such as Jussila et al. (1992) or Dumlupinar et al. (2011).

Unfortunately, this assumption cannot be applied to all examined varieties. For example, USA/Canadian varieties were slightly diverse than the varieties mentioned above but less diverse than Czech varieties. However, according to Rodger et al. (1983), the North American varieties are reaching a very high degree of genetic diversity, compared to European ones, because of the frequent use of exotic varieties in breeding programs, which was also confirmed later by Fu et al. (2005) and Achleitner et al. (2008). Besides that, many North American varieties have an ancestor from Russia (variety Kherson) and contain germplasm of *A. byzantina* (Coffman 1977).

Czech varieties were evaluated as the most diverse ones, which was demonstrated by their polymorphic avenin spectra and visualized on the dendrogram layout, since they were distributed across all six clusters, however, the highest quantity of Czech varieties was observed in cluster 1 and 3. Interestingly, the majority of them originated from the same breeding program Selgen Krukanice, a.s., Czech Republic. On the other hand, it should also be taken into account that the Czech varieties were the most represented in this study, compared to the other samples of different origin.

Only a few of the Czech samples were clustered with another Czech variety, such as Radius – Otakar, Sagar – Atego, SG-K16654 – Kertag, SG-K6027 – Tibor, Vok – Gregor, and Cyril – Norbert. Similar results, based on Identity index calculation, were observed in the study of Polišenská et al. (2011), where some avenin patterns resulted in a high identity index, whereas some reached low values. Dvořáček et al. (2003) achieved similar results, where some of the varieties clustered, but some of them remained separated. However, these results may be affected by a relatively low number of analyzed samples – 49 analyzed samples by Polišenská et al. (2011) and five varieties by Dvořáček et al. (2003).

The two most distant Czech varieties, Radius and Dalimil, shared only one avenin band position; thus, they were also analyzed according to their pedigree and ancestors. Only two identical varieties were used during the breeding process – Unnamed_7766 and Ardo (Figure 17). Therefore, it can be assumed that the distant genetic background may result in distant avenin phenotypes (Jussila 1992; Portyanko et al. 1998).

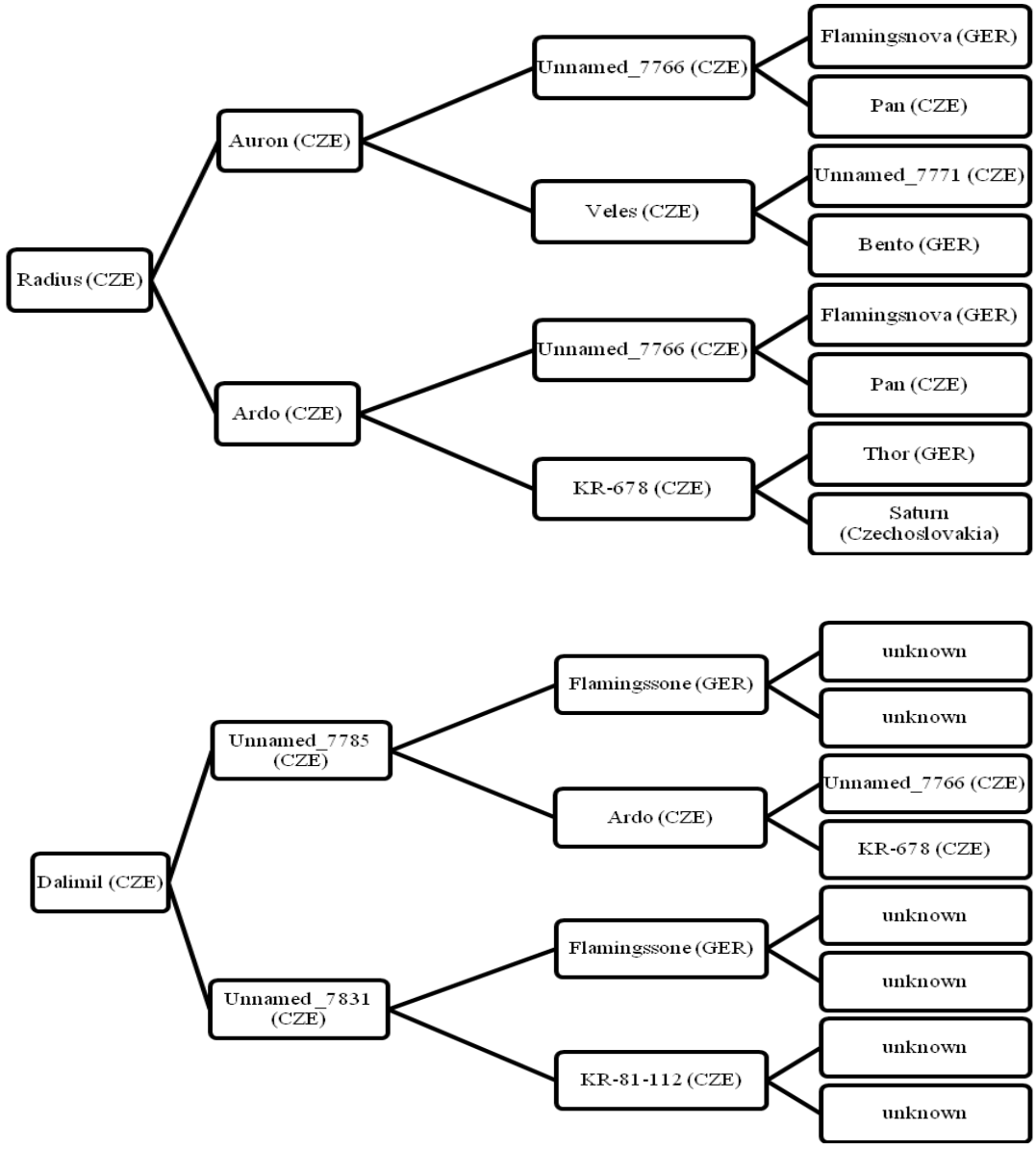


**Figure 17.** Pedigree of the two most distant Czech varieties Radius and Dalimil (POOL 2013)

Varieties labeled as tropical or subtropical resulted in a relatively similar pattern to the Czech varieties because they were also assembling in clusters 1 and 3. More surprisingly, five of these tropical/subtropical varieties were somehow related to Czech varieties, according to the avenin band pattern. For a better understanding of the varietal relationship, the pedigree comparison should be performed; however, it was impossible to trace back the ancestors of analyzed tropical/subtropical samples, since their pedigree was unknown or not available at all.

The two varieties of distant origin were, for example, Klein 69B (Argentina) and Izak (Czech Republic) that shared five similar avenin band position. Klein 69B was bred by the selection from common yellow oats from Argentina, impossible to trace back any other ancestors, whereas Izak was bred mostly from Czech varieties and to a lesser extent from German and Netherland varieties (POOL 2013).

Although economically essential species – *A. sativa* and *A. byzantina* were introduced to Latin America from Europe in the history (FAO 2004); according to Martinuzzi & Carbajo (1978), the most popular recent cultivar in Argentina was Suregrain with ancestors originating in the USA and also Algeria (POOL 2013). This fact is also supported by the start of an international breeding program in 1974, run by USAID (U.S. Agency for International Development) and later by Quaker International Oat Nursery, that developed and released several oat varieties in Argentina, Brazil, Chile and Uruguay (FAO 2004).

## 5.4. Reactivity of Oats Related to Avenin Band Spectra

Oat prolamins – avenins – are considered as a trigger of a toxic response in some patients having CD, autoimmune enteropathy causing inflammation of the small intestine and villous atrophy (Arentz-Hansen et al. 2004; Rostom et al. 2006; Kagnoff 2007; Gregor et al. 2010; Tuire et al. 2012). The research showed that gliadin-like reactive peptides cause the toxicity in avenins; however, their amount is variable and strongly depends on the variety (Comino et al. 2011; Ballabio et al. 2011; Mujico et al. 2011).

Oats are generally referred as non-immunogenic or less immunogenic than other cereals (Radish et al. 2007; Ellis & Ciclitira 2008; Cooper et al. 2013), because the amount of prolamins is much lower, around 4 – 15 % of total oat protein (Lásztity 1998;

Peterson 2011, Klose & Arendt 2012), compared to wheat, barley or rye (Haboubi et al. 2006). However, there is a gluten threshold of 20 mg/kg (20 ppm) for gluten-free labeling in Europe and the USA based on the Codex Alimentarius standard (Commission Implementing Regulation 828/2014 2014).

According to previous research, the reactive proteins appeared to have a molecular weight range of 25 – 37 kDa (Mickowska et al. 2015; Comino et al. 2016), which corresponds to bands investigated in this study, that were marked as no. $1^{+}$ to 6* (25.0 – 30.2 kDa). The frequencies reached by avenin bands in this spectrum were rather moderate to low compared to the remaining region. Also, frequencies of individual seed samples and bulk samples differ in some bands. This difference could be the result of intra-varietal polymorphism or the lower number of analyzed samples examined by the single seed analysis.

In our investigations, three varieties contained only one band in the reactive protein spectrum - Maelor (GBR), Shadow (CAN), and Troshaver uit Besel (NLD). The testing by Gluten G12 Sandwich ELISA confirmed that variety Shadow contains less than five ppm of reactive gluten (Romer Labs 2015). Mujico et al. (2011) analyzed 26 different varieties of origination in the Netherland, some of which were also included in this study. Although Troshaver uit Besel was evaluated as potentially toxic for CD patients, varieties Wodan and Zandster were found to have the minimal stimulatory capacity, thus minimum reactive epitopes.

Nevertheless, those two varieties had two (Wodan) and three (Zandster) avenin protein bands in the reactive protein spectrum in this research. On the other hand, potentially reactive varieties were Black, CC4146, Sang, and Sirene, since they contained six protein bands in the reactive spectrum. However, there is not any scientific evidence about their reactivity or non-reactivity; thus, western blot and ELISA analysis are recommended for further research.

# 6. Conclusions

In this study, the avenin analysis of 151 oat samples was performed, with a result of 26 allelic avenin positions in the molecular weight range of 18 to 35 kDa.

Out of 127 oat varieties that were examined by a single seed method, 29% of samples showed intra-varietal polymorphism. The number of bands oscillated between 6 – 10 bands in non-variable single seed samples and 5 – 10 bands in variable single seed samples. The allelic position with the highest frequency of occurrence was band no. 8 (23.5 kDa).

A total of 151 oat varieties were analyzed as the bulk samples. The total number of bands per sample ranged from 5 – 11 bands. The allelic position with the highest frequency was the same as in single seed samples. There was no intra-varietal polymorphism in bulk samples; thus, it is recommended to use this type of method for avenin band analysis. The results obtained in this study disprove the hypothesis that there is no difference in allelic variability among single seed and bulk sample analysis.

Altogether, there were three varieties in each type of avenin spectra analysis that might have the potential to be low in reactive peptides, thus suitable for CD patients, since those varieties had only one protein band lying in the reactive protein band spectra.

Based on the analysis of varieties´ origin, it cannot be proved that the geographical origin affects the avenin variability. A relatively similar pattern of the avenin band spectrum was observed in Austrian and French varieties. The highest avenin polymorphism was evaluated for the Czech varieties. Varieties originating in tropical/subtropical countries were also polymorphic, but their avenin band pattern showed some similarities with European and Canadian varieties.

The dendrogram obtained through the avenin band pattern comparison was divided into six main clusters. The least consistent cluster was cluster 1, whereas the most consistent was cluster 6. The inter-varietal polymorphism of studied samples obtained through SDS-PAGE separation was high since there was no similar avenin band pattern; therefore, the second hypothesis was confirmed.

It can be concluded that an avenin separation is a useful tool for varieties identification. Moreover, the potential of high polymorphism in the protein band pattern gives the possibility of reducing the number of proteins that are harmful to CD patients.

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# Appendices

**List of the Appendices:**

# Appendix 1: The list of varieties divided into six clusters with the distribution corresponding to the dendrogram results

The layout of each cluster corresponds to the dendrogram results (Figure 9), which was created based on the genetic relationship of 151 examined bulk samples and calculated by using Darwin software. The origin of the varieties is included and marked by colours.

**Blue –** Czech varieties, **orange –** German varieties, **red –** Great Britain and Ireland varieties, **yellow –** French varieties, **brown** – Austrian varieties, **pink –** North American varieties, **green –** remaining non-European countries (Argentina, Australia, India, Morocco, South Africa), **black –** remaining European countries (Belgium, Denmark, Estonia, Finland, Hungary, Italy, Netherland, Norway, Poland, Serbia and Montenegro, Sweden) and Russian varieties.

**Cluster 1**

| |
|---|
| Radius (CZE) |
| Otakar (CZE) |
| Buddah (AUS) |
| Celeste (CZE) |
| APR166 (UNKNOWN) |
| SG-K 16370 (CZE) |
| Martin (NOR) |
| Jongensklip (ZAF) |
| Euro (AUT) |
| SG-K 16658 (CZE) |
| Dominik (GER) |
| Pennlo (USA) |
| Merlin (CZE) |
| Rouge 31 (DZA) |
| Oliver (CZE) |
| Seldon (CZE) |
| Drummer (GER) |
| Abel (CZE) |
| Sagar (CZE) |
| Atego (CZE) |
| Valiant (NLD) |
| Mojacar (CZE) |
| Auron (CZE) |
| Pusahybrid (IRL) |
| Milton (USA) |
| Efesos (AUT) |
| Český žlutý (CZE) |
| Dukat (POL) |
| AC Preaknes (CAN) |
| Kanota (USA) |
| Raven (CZE) |
| Ogle (USA) |
| Rogar 8 (ITA) |
| Jaak (EST) |
| Salo (SWE) |
| Tucana (AUS) |
| OA 504-5 (CAN) |
| Neklan (CZE) |

**Cluster 2**

| |
|---|
| KWS Contender (GER) |
| Roope (FIN) |
| Garland (USA) |
| Lennon (GBR) |
| Freddy (GER) |
| CC4146 (GBR) |
| La Prevision 7 (ARG) |
| Ardo (CZE) |
| Kalle (EST) |
| Johanna (BEL) |
| Precoce de Maroc (MAF |
| Zuton (GBR) |
| Avenuda (CZE) |
| David (CZE) |
| Kamil (CZE) |
| Black (FRA) |

**Cluster 3**

| |
|---|
| Mediteran (SCG) |
| Dakar (FRA) |
| Cavaliere (CZE) |
| Ranch (FRA) |
| Auteuil (FRA) |
| Astor (NLD) |
| Banquo (GBR) |
| Maldwyn (GBR) |
| Sirene (FRA) |
| Patrik (CZE) |
| Max (DNK) |
| SG-K 16654 (CZE) |
| Kertag (CZE) |
| Bundy (AUS) |
| SG-K 16472 (CZE) |
| Marco Polo (CZE) |
| Flamingsnova (GER) |
| Zandster (NLD) |
| Maelor (GBR) |
| Troshaver uit Besel (NLD) |
| Coach (GER) |
| Matilda (SWE) |
| NP1 (IND) |
| Weston II (IND) |
| SG-K 16562 (CZE) |
| Polaris (ARG) |
| Maris Oberon (GBR) |
| Melys (GBR) |
| Jawor (POL) |
| Rozmar (CZE) |
| Husky (GER) |

**Cluster 4**

| |
|---|
| SG-K 6027 (CZE) |
| Tibor (CZE) |
| Aragon (GER) |
| Yty (FIN) |
| Nordstern (GER) |
| Pan (CZE) |
| Debyut (BEL) |
| Avesta (FRA) |
| Adam (CZE) |
| Klein 69 B (ARG) |
| Izak (CZE) |
| Vok (CZE) |
| Gregor (CZE) |
| Katri (FIN) |
| Walderm (CAN) |
| Flämingsprofi (GE |
| CDC Boyer (CAN) |
| Belinda (FIN) |
| Azur (CZE) |
| Otee (USA) |
| GK Iringo (HUN) |
| Aarre (FIN) |
| Classic (USA) |
| Sang (SWE) |
| Veli (FIN) |
| Hynek (CZE) |
| SG-K 16564 (CZE |
| Canyon (GER) |
| Gambo (NLD) |

**Cluster 5**

| |
|---|
| Norbert (CZE) |
| Cyril (CZE) |
| Longchamp (FRA) |
| Veles (CZE) |
| Ivore (FRA) |
| Shadow (CAN) |
| Diadém (CZE) |
| Saul (CZE) |
| NP2 (IND) |
| Kermit (IRL) |
| Riel (CAN) |
| Florian (CZE) |
| Gana (RUS) |
| Buggy (GER) |
| Wodan (NLD) |
| Expo (AUT) |
| Apollon (RUS) |
| Mansholts III (NLD) |
| Poseidon (GER) |
| Prairie (USA) |

**Cluster 6**

| |
|---|
| Tjumenski golozernõi (RUS) |
| Myriane (NLD) |
| Lidya (RUS) |
| Korok (CZE) |
| Santini (CZE) |
| Salomon (GER) |
| Poncho (FRA) |
| Dalimil (CZE) |
| Expander (AUT) |
| Espresso (AUT) |
| Efendi (AUT) |
| Ebene (FRA) |
| Navaro (CAN) |
| Bison (RUS) |
| Jim (USA) |
| Leo (GER) |
| Scorpion (GER) |