

UNVEILING THE MESS OF RED POTTAGE THROUGH GEL ELECTROPHORESIS: A ROBUST AND RELIABLE METHOD TO IDENTIFY *VICIA SATIVA* AND *LENS CULINARIS* FROM A MIXED LOT OF SPLIT “RED DAL”

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Abstract

Due to similarity in seed texture, colour and size of vetch (*Vicia sativa*) with lentil (*Lens culinaris*), these two legumes are mixed when split as “dal” to fetch higher prices. The sole marketing as split seed of vetch cultivar “Blanche fleur” under the false name of “red dal” or its mixing with lentil created hue and cry during the last decade of previous millennium in most of the South Asian countries including Middle East. Identification of vetch from lentil was only possible through modern biochemical techniques involving sophisticated equipment and technical skill. One hundred and ten samples of split red dal along with reference sample for both the species collected from various grain markets were analyzed through SDS-PAGE technique for three times, starting in 2001 with the interval of five years. The seed protein profiling was employed for distinguishing these two legumes belonging to different genera that were successfully utilized and the information was shared with researchers to create awareness among the consumers through print media. Varying degrees of mixing was observed that was gradually decreased during second and third sampling phase which was mainly due to robust information generated and dispersed. The electrophoretic pattern indicated a clear-cut differentiation of *V. sativa* from *L. culinaris*, hence this technique is very effective for species identification.

Introduction

Lentil (*Lens culinaris* Medik.) is a cool season pulse crop consumed in one or the other way through out world (Asim, 2012) and the International Centre for Agriculture in Dry Areas (ICARDA), in Syria, has world mandate of this crop for breeding and genetic improvement. In many south Asian countries it is being consumed as split “dal” and when it is split, the cotyledon color varies from yellow to orange, later is preferred in many South Asian countries. Common vetch (*Vicia sativa* L.) is another winter season legume commonly used for ruminant animals. Both of these species have been reported to be utilized by man during early ages (Ladizinsky 1979a; Melamed *et al.*, 2008). This species contain nine toxic compounds including neurotoxin, β -Cyano-L-alanine that can affect non-ruminants including monkeys, pigs, mules, horses and poultry birds (Ressler *et al.*, 1969). Vetch has been cultivated for centuries as a forage legume worldwide and similarly in Pakistan. It also co-exists as weed in the lentil fields and remains in low quantities in the lentil seed lots either in whole or split seeds (Lambein *et al.*, 2005). Australia and China being the leading countries for vetch production export to many developing countries, with labels “not for human consumption”. Development of new vetch varieties including “Blanche fleur” with red cotyledon enhanced the production as cash-crop in Australia during eighties increased export of vetch seed considerably (Tate & Enneking, 1992). The spherical cotyledons of the Blanche fleur cultivar of vetch are morphologically similar to the split red lentil, particularly when coated with vegetable oil. Feeding on vetch for a month cause toxic effects due to neurotoxins (β -cyanoalanine and γ -glutamyl, β -cyanoalanine) to the animals and if more than 10% vetch is mixed with lentil it cause toxicity for humans.

During the decade ending 2000, there have been several incidences of split red vetch being exported from Australia, relabeled and sold at a higher premium as split red lentil (red dal) for human consumption in Pakistan. Consequently upon public concern regarding the neurotoxic effects of vetch, a temporary ban was observed on imports of Australian vetch into several countries, including Egypt, India, Pakistan and Bangladesh (Tate *et al.*, 1999). Due to hue and cry on the mixing of vetch and lentil as split dal, there was a need to have a quick and robust methodology to identify both of these species from the mixed lots. Although methodologies have been developed to distinguish between different species on the basis of isozyme polymorphisms in *Cicer* (Labdi *et al.*, 1996; Sudupak & Kence 2004), in *Lens* (Hoffman *et al.*, 1986), radish (Jatoi *et al.*, 2011) and *Vicia* (Mirali *et al.*, 2007; Oujii *et al.*, 2011). The seed protein profiling resolved through SDS-PAGE has been successfully used for determining inter-specific diversity in legumes mainly in *Vigna* (Ghafoor *et al.*, 2002), in *Lathyrus*, (Emre, 2009), in *Lens* (Ladizinsky, 1979b), in *Pisum* (Jha & Ohri, 2002), *Vicia* (Haider & El-Shanshoury 2000; Berger *et al.*, 2002; Emre, 2011), and in *Cicer* species (Ahmad & Slinkard, 1992).

Vetch also can be identified biochemically by measuring the β -cyanoalanine content using chromatographic procedures that are time consuming and laborious and that rely on the availability of a polyclonal antibody (Ressler, 1962) or through a PCR-based marker sequence tagged microsatellite site (STMS) markers (Pandian *et al.*, 2002). These techniques employ either highly equipped laboratory facilities with high levels of skill or these are expensive, especially for the countries where the modern tools of biotechnology are not established properly (Rehman *et al.*, 2013). Due to high degree of specificity of seed proteins for closely related species, it was planned to have a robust and reliable

methods to identify vetch and lentil species even on single split seed that could also be used with minimum level of knowledge and technology. Therefore this research was initiated to identify lentil and vetch from mixed lots and the results are likely to be utilized both for genebank management and for commercial marketing to safeguard consumers' rights.

Materials and Methods

During 2001, 110 samples of split red dal were collected from various grain markets including one sample of whole lentil and one of vetch from each collection site as a reference for both the species. The number of samples and the collection sites are presented in the Fig. 1. To assess the usefulness of awareness campaign and the validity of the technique for identification of species specific proteins, the same sites were re-sampled after an interval of five years, *i.e.*, during 2006 and 2011, respectively. For all the 110 samples during all the times, 20 seeds per sample were analyzed for seed proteins using SDS-PAGE to assess the mixing of these legumes. For all the samples, single seed was ground to fine powder with mortar and pestle for the extraction of proteins. Sample buffer (400 μ L) was added

to 0.01 g of seed flour as extraction liquid and mixed thoroughly in Eppendorf tube with a small glass rod. The extraction buffer contained the following final concentrations: 0.5 M Tris-HCl (pH 6.8), 2.5% SDS, 10% glycerol and 5% 2-mercaptoethanol. Bromophenol Blue (BPB) was added to the sample buffer as tracking dye to watch the movement of protein in the gel. Seed protein was analyzed through slab type SDS-PAGE using 11.25% polyacrylamide gel. The gel size was 12.0 x 13.8 cm². In order to check the reproducibility of the method, two separate gels were run under similar electrophoretic conditions. The SDS-PAGE of total seed protein was carried out in the discontinuous buffer system according to the method of Laemmli (1970). After staining and destaining the gels, depending upon the presence or absence of polypeptide bands. The data were presented and discussed qualitatively on the basis of adulteration of lentil with vetch. Most of the samples were unable to identify visually from the red dal samples, whereas based on SDS-PAGE for seed proteins the mixing was observed for various collection sites in varying degrees. As identification of vetch was not possible on visual morphological diagnostic traits of split seed, the seed proteins successfully differentiated vetch from lentil even when split.

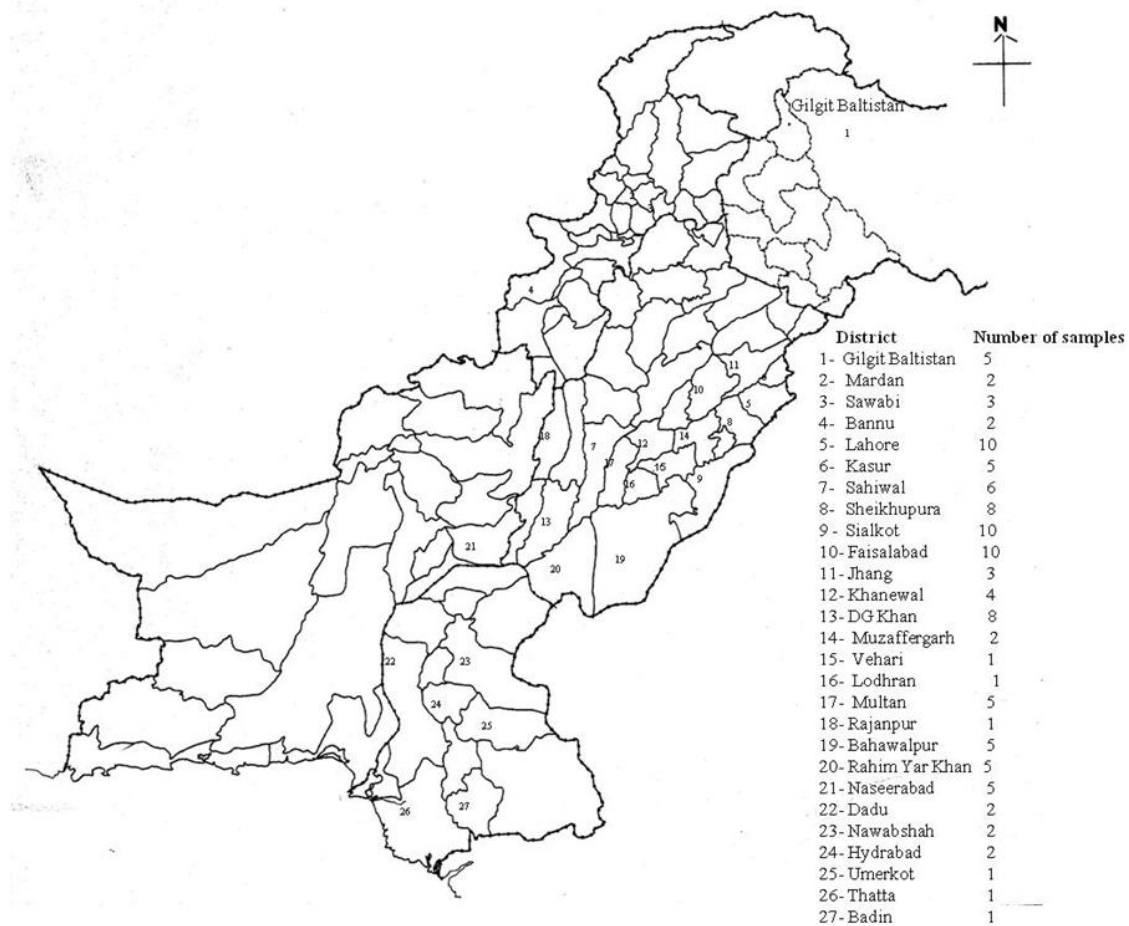


Fig. 1. Map representing the locations for collection of samples of "red dal" for analysis by SDS-PAGE technique. The districts are marked in the map as numeric value and listed along with number of samples collected from particular district.

Result and Discussion

The electrophoretic pattern indicated a clear-cut differentiation for *L. culinaris* and *V. sativa* (Fig. 2). Three distinct bands were observed consistently in the vetch samples and the same bands were used reference for distinguishing vetch from lentil. The mixing percentage was calculated accordingly. Rosa & González (2010) characterized vetch on the basis of seed storage proteins and detected 49 polymorphic bands for intra-specific variation, whereas in the present study intra-specific variation was not prominent, and was not considered as the main purpose of this study to identify vetch and lentil from mixed population, especially when these were split. The variation was observed on the basis of SDS-PAGE in lentil that has been published by Sultana *et al.*, (2006), which reported diversity for seed protein profiles in 144 indigenous lentil germplasm, whereas in another study they identified landraces from Pakistan (Sultana & Ghafoor, 2008). Emre (2011) reported inter-specific and intra-specific variations in nine *Vicia* spp., using SDS-PAGE and observed that nine taxa were clearly identifiable from the protein patterns. Valizadeh (2001) investigated differences among 11 legumes species on the basis of SDS-PAGE and considered this technique efficient procedure for differentiating grain legume species. In the present study, intra-specific diversity for vetch was not much conspicuous that might be due to one prominent vetch variety that was imported in bulk quantity and perhaps mixed in lentil. Although vetch is being cultivated in Pakistan, but not included in the investigation because the objective of this study was to distinguish two species from mixed red dal rather than to investigate intra-specific diversity in either of the legumes. Further the local vetch has yellow cotyledon colour, hence has no scope of mixing in orange lentil dal.

One hundred and ten samples collected after the interval of five years, starting from 2001, were analyzed

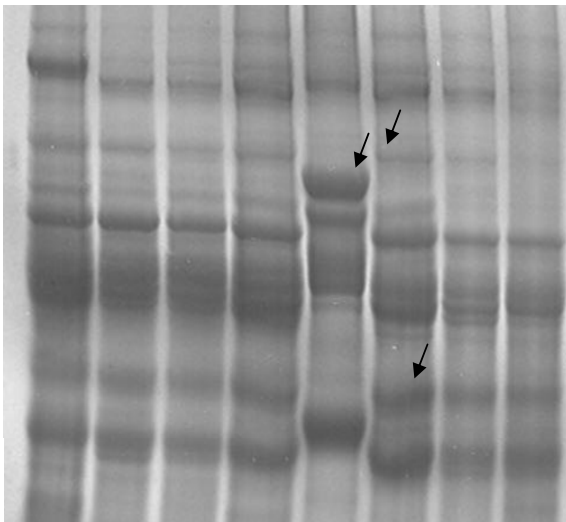


Fig. 2. Identification of *Lens culinaris* (lentil) and *Vicia sativa* (vetch) from a mixed lot when split dal. The arrows represent the protein specific fragments for vetch.

for seed protein profiling that indicated varying degrees of mixture during different collection regimes (Fig. 3). The samples collected during 2001, 2006 and 2011 indicated that five samples were pure lentil in the samples collected during 2001, whereas 38 samples were mixed with > 20% of vetch seed. Mixing of vetch in lentil fields had been listed since primitive era, but Vavilov (1922) illustrated and published both of these species similar as colour plate for his law on homologous series of variation in plants. The law of homologous series might have the basis described by Thissen (1999) who believed that during the neolithisation period the cooking of lentils and vetch was major food stuffs at early Ilipinar (38° 41' 24" North, 26° 55' 00" East) during prehistoric era. The close resemblance between red lentils and the *Vicia sativa* cultivar "Blanche fleur" further led to its marketing for human consumption during the early 1990s that was readily taken up by the concerned countries and the researchers established various techniques for separating these two similar legumes and stopped mixing of vetch in lentils. During the last decade of previous century, the considerable amounts of common vetch (cv 'Blanche fleur') destined for substituting split red lentils or mixed with lentil in markets of the Middle East and the Indian Subcontinent that was a serious cause for concern at that time (Tate & Enneking, 1992). During five years interval after the first analyses, decrease in mixing was observed and six samples were observed mixed with > 15% of vetch, whereas clean lentil samples were doubled during first five years interval and more than 90% samples were pure lentil as analyzed during 2011. Seven samples were observed mixed with vetch ranging from 0.2 to 2% in the samples collected during 2011. The awareness among the researchers and consumers helped in avoiding mixing of vetch in lentil and the consumers were safeguarded as they get what they pay.

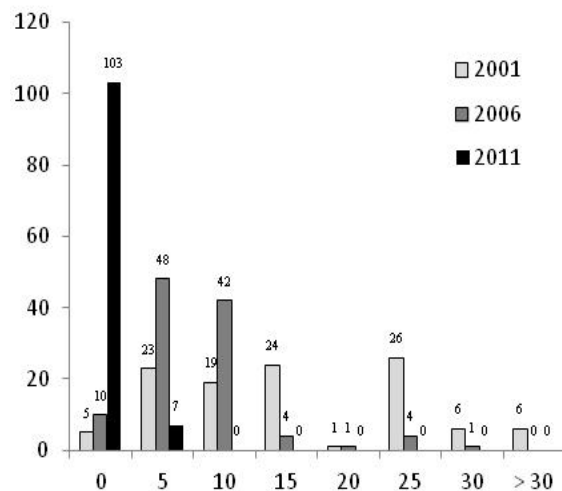


Fig. 3. Mixing of vetch in lentil as revealed through seed protein profiling during 2001, 2006 and 2011 analyzed by SDS-PAGE technique.

Co-existence of lentil and vetch has been reported in archeological studies (Barrett 1983; Erskine *et al.*, 1994) that is still continuingly occurring under farmers' fields of most of the Asian countries and very limited quantity is expected as weedy nature of vetch, but due to mimicry of lentil with similar legumes, the missing of vetch instigated the researchers to resolve the issue to clean up the lentil market from mixing, especially with vetch that is toxic and not fit for human consumption. Pandian *et al.*, (2002) suggested a PCR-based marker system for identification of morphologically similar split seed of vetch and lentil using sequence tagged microsatellite site (STMS) markers and a STMS primer-pair (*PSMPSAD123*) was able to separate split red cotyledon vetch from split red cotyledon at a specific markers at 563 bp for lentil and 353 and 474 bp for vetch. Food security along with authenticity is the prime concern of the consumers and is a hot topic of the present era in food analysis (Virginia & Cifuentes 2008). A common problem in grain legumes is the replacement of high-quality seeds by other seeds with high resemblance, but that can be toxic or to present poorer quality (Cifuentes, 2006). The case for lentil that often is contaminated by seeds of vetch is still a problem in many countries, especially developing ones. Piergiovanni & Taranto (2005) observed the practice of mixing *V. sativa* and *V. articulata* resembling with small-seeded lentil cultivars and reported capillary electrophoresis method for the differentiation of lentil cultivars from the vetch to which he called false lentil. The proposed methodology in this paper is simple and not affected by environments, protocol is readily available that does not require high technical skill, hence can be performed with minimum labour and time to get reliable results, especially in developing countries and the information will be helpful to safeguard consumers' rights.

Conclusions

The SDS-PAGE performed on single split seed using 11.25 percent gel indicated the specific protein bands for vetch those could be used to distinguish lentil from vetch. Split dal of lentil or similar to lentil is recommended to test prior to market for human consumption. The seed proteins are conservative in nature and proved its worth for distinguishing two species. The assay is easy to analyze even from half seed. The awareness among the researchers and consumer caused significant improvement in lentil market and the mixing was eliminated within one decade. The consumers' rights were safeguarded as to get the same for which they pay. The technique is robust, simple, and reliable, requires minimum biotechnological skill and can be conducted in an ordinary graduate laboratory in any developing country. The consecutive analyses for three times with five years interval indicated gradual decrease in mixing vetch with lentil that was possible through SDS-PAGE analyses and continuous campaigns against malpractice of adulteration of two similar legumes.

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