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Postal Code: 9177948953
P.O. Box: 917751436
Tel: +98-513-8804063
Fax: +98-513-8795162
E-mail: jcmr@um.ac.ir
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The Editorial

Molecular Interactions of lncRNAs: Cellular Fate Determination and Tissue Regeneration

Monireh Bahrami [1,2*] and Muhammad Irfan-Maqsood [1,2]

[1] *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Editorial Management Office, Journal of Cell and Molecular Research, Ferdowsi University of Mashhad, Mashhad, Iran*

Summary

LncRNAs (long non-coding RNAs), defined as non-coding RNAs with length ≥200 nt, are responsible to control the degradation process, RNA stability, orchestration, inhibition, transcription and histone modification etc. These RNAs have been termed as the key agents of several vital mechanisms such as development, organogenesis and regeneration of damaged tissues etc. They interact with a number of partner molecules either proteins, RNAs or DNAs. They also control the cellular behaviour of stem cells such as differentiation or self-renewal or their paracrine effects. This editorial is discussing the significance of lncRNAs as therapeutic target in stem cell therapy field.

***Keywords*:** LncRNAs, Regeneration, Molecular interactions, Stem cell fate, Differentiation

LncRNAs (long non-coding RNAs) are defined as the transcripts of greater than 200 nucleotides that lack ORF (open reading frames) and perform a lot of important functions other than coding proteins. RNA polymerase II transcribe them and then they spliced and polyadenalated (Rinn and Chang, 2012). It has been discovered that they are involved in the generation of variety of other nucleic acids such as miRNAs, other-ncRNAs etc (Ogawa et al., 2008; Rogler et al., 2014). LncRNAs have been found to be involved in many known process with interaction of other molecules such as, they are involved in (1) the degradation process when interact with miR-9 (Leucci et al., 2013), (2) enhanced BACE mRNA stability while interacting with miR-485-5p (Faghihi et al., 2010), (3) participates in the orchestration of an intrachromosal loop while interacting with RUNX1 promoter and enhancers (Wang et al., 2014), (4) tether with DNA to recruit inhibitor proteins (Wang et al., 2008), (5) dissociate the preinitiation complex when bind with DHFR promoter (Ponting et al., 2009), (6) form histone modification complex by bridging with PRC2 and the lysine demethylase LSD1 (Tsai et al., 2010), (7) activate the *Dlx5/6* enhancer when cooperate with *Dlx2* homeodomain protein (Feng et al., 2006) and etc.

It has been confirmed that lncRNAs control some vital functions in the development, organogenesis and regeneration of damaged tissues. Their role has been identified in the differentiation and terminal differentiation of somatic stem cells to improve wound healing in traumatic injuries (Beasley et al., 2015; Kretz et al., 2013). It has been discovered that these lncRNAs are involved in the maintenance of stem cells state and to determine the stem cell fate, which lineage it has to adopt and either it has to differentiate or terminally differentiate. As we have discussed here they have very strong molecular interactions with almost all kind of RNAs, DNAs and proteins to play their vital role in cellular behaviour. Every lncRNA has its own specific molecular partners where they interact each other and control the cell fate. A number of cellular mechanisms controlled by lncRNAs in coordination with their partner molecules have been shown in table 1.

Table 1. LncRNAs and their interacted partner molecules to control cellular fate (Flynn and Chang, 2014).

Sr. No.	LncRNA	Partner Molecules	Targeting Cells
1	TUNA/megamind	PTBP1, NCL, hnRNP-K	Neuronal Cells
2	Dix1as	?	Neurons
3	Six3os1	?	Oligodendrocytes
4	TINCR	STAU1	Differentiated Keratinocytes
5	ANCR	?	Skin Stem Cells
6	Braveheart	PRC2	Cardiocytes
7	Fendrr	PRC2 or MLL	Cardiac and Lung Cells
8	Yam1	YY1	Muscle Stem Cells
9	Linc-MD1/miR133	AGO and HuR	Muscle Tisses

∗Corresponding author E-mail: *jcmr@um.ac.ir*

Considering the molecular interactions of lncRNAs and their role in stem cell fate determination and to regulate tissue regeneration, it can be concluded that lncRNAs have been named as the centrally controlling molecules for tissue regeneration. Full and detailed networking maps of these lncRNAs should be discovered so that these RNAs can be used as targeted therapeutic agents in clinics.

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Review Article

The Role of Mesenchymal Stem Cells in Skin Wound Healing

Tahereh Sanjari [1], Toktam Hajjar [1], Madjid Momeni-Moghaddam [1*]

[1] *Department of Biology, Faculty of Sciences, Hakim Sabzevari University, Sabzevar, Iran*

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Abstract

Mesenchymal stem cells (MSCs) could differentiate into various types of tissues. These cells serve as a backup for the regeneration and repair of tissues or cells after injury. A skin wound is defined as an injury to the skin that needs to be restored. All types of cells in skin especially mesenchymal stem cells play important role in wound healing process. In particular, paracrine signaling of MSC regulates the cellular responses at the wound site leading to reduction of inflammation, stimulation of angiogenesis and induction of cell migration and proliferation. Because of these abilities, MSCs are one of the most common stem cells for cell therapy in wound healing. This review focuses on the role of MSCs on wound healing process. In addition, major phases of wound repair and challenges of cell therapy are discussed.

***Keywords*:** Mesenchymal Stem Cells, Wound healing, Inflammation, Angiogenesis

Introduction·

Mesenchymal stem cells have been characterized to be cells mainly responsible for the repair of damaged tissues (Ai et al., 2002). MSCs are multipotent stem cells that play crucial roles in the maintenance and repair of tissues; these cells are essential for wound healing (Nuschke, 2014) . In addition, MSCs promote regulation of immune response and inflammation as well as induction of cell migration and epithelial changes (Maxson et al., 2012). These abilities of MSCs and the development of tissue engineering and production of cellular scaffolds, have been introduced as a new strategies in the treatment of chronic wounds (Nuschke, 2014).

The skin is the largest organ of the body and contains a large number of MSCs (Li et al., 2006). Since most important function of skin to disease control is the formation of a physical barrier against pathogen factors, any factor that would break the barrier can produce lesion and will ultimately weaken the body, so wound healing emerges as an alternative mechanism to the treatment of injured tissues (Singer and Clark, 1999). Recent studies indicate that skin stem cells participate broadly in dermal repair during healing of skin legions particularly chronic diabetic foot ulcer (Kato et al., 2014). Although wound is generally defined as cut and severe damage, a wound by true definition is an injury or disruption in normal skin structure and function. It can be included a wide range of damage, ranging from a simple epithelium injury to deep damage involving tissues below the level of the skin and even tendon, muscle, vessels, nerves and bones (Velnar et al., 2009). Skin legion treatment is a diverse part of the health care system, including surgical and accidental lacerations, burns, pressure ulcers, diabetic and venous ulcers (Chen et al., 2009).

After the injury, the body organizes programmed repair process called wound healing. Wound healing is a complex and multistage process in which damaged skin is repaired (Velnar et al., 2009). This review considers function of MSCs on wound healing as well as main phases of the process and ongoing challenges in cell therapy.

Wound Healing Phases

Wound healing can be divided into several phases which must occur in a specific sequence at a specific time and each phase must continue for a specific duration at an optimal intensity (Guo and DiPietro, 2010). Thus, wound healing process involves four phases: hemostasis, inflammation, proliferation and remodeling (Bielefeld et al., 2013).

Corresponding authors E-mail:
** iranbioman@yahoo.com*

Hemostasis

When tissue is injured, the small blood vessels suffer from damage. This condition can lead to bleeding and body momentarily stops loss of blood, a process called hemostasis (Young and McNaught, 2011). In this phase, vasoconstriction occurs before activation of platelets and coagulation. The endothelium of damaged vessels produces a vasoconstrictor, endothelin, which leads to vasoconstriction. Other mediators for vasoconstriction are derived from sympathetic nervous system (norepinephrine), the circulating catecholamine (epinephrine), and the release of prostaglandins from injured cells (Teller and White, 2011). Vasoconstriction of damaged blood vessels will cause a hypoxic microenvironment within wound area that leads to production of reactive oxygen species (ROS). It can enhance expression of antioxidant enzymes to detoxification of excess ROS (Behm et al., 2012). At this stage, blood loss is also prevented through the formation of a clot (Young and McNaught, 2011).

The coagulation cascade is made up of two converging pathways: extrinsic and intrinsic. Although both pathways start in different ways, each of them leads to the activation of factor X and the production of thrombin. Thrombin plays two important roles in clot formation: a catalyst for the conversion of fibrinogen to fibrin and an originator for platelet activation (Teller and White, 2011). Platelets have also important role in this phase. Platelets contact with collagen of the damaged vessels and thrombin which leads to their activation (Olczyk et al., 2014). Activated platelets adhere at site of exposed collagen to form a platelet plug and temporarily stop bleeding (Young and McNaught, 2011). Moreover, the blood clot contains fibrin, fibronectin, vitronectin and thrombospondins that create a temporary matrix, which serve as scaffold to migration and adhesion of fibroblasts, keratinocytes, and endothelial cells (Olczyk et al., 2014; Reinke and Sorg, 2012).

The aggregated platelets, trapped in the temporary matrix, release various growth factors, such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), and basic fibroblast growth factor (bFGF) from α granules. These mediators influence neutrophils, monocytes, macrophages, smooth muscle cells, and fibroblasts (Olczyk et al., 2014).

Inflammation

This stage is accompanied by specific inflammatory symptoms, such as redness, body heat, swelling, and pain around the wounded place. In the early inflammation phase, with subsiding of the initial vessel contraction, vascular permeability of walls increases by factors such as histamine, kinases, prostaglandins, leukotrienes, hyaluronic acid and ROS (Olczyk et al., 2014).

Neutrophils are the first subset of leukocytes to enter the wound and within 24 hours will become the dominant neutrophils in wound area. The presence of neutrophils stimulated by prostaglandins, complement, TGF- β, tumor necrosis factor alpha (TNF-a), Interleukin-1 (IL-1), and bacterial products at the wound site. Neutrophils release various types of proteolytic enzymes, which break down bacteria and extracellular matrix (ECM) within the injury area as well as produce reactive oxygen free radicals to prevent microorganism's penetration (Teller and White, 2011; Werner and Grose, 2003).

When neutrophils curried out their tasks, these specific cells must be eliminated from the wound by either apoptosis or macrophage phagocytosis (Olczyk et al., 2014; Teller and White, 2011; Young and McNaught, 2011).

After 48 to 96 hours, the predominant leukocyte within a wound is the macrophage (Teller and White, 2011). Macrophage play several function within wound site including: host defense, promote inflammation, removal of dead cells, supporting cell proliferation and tissue repair. Along with the immunological function, macrophages play crucial roles in cell proliferation and synthesis of extracellular matrix component of skin cells by production of TGF-α, TGF-β, bFGF, PDGF, and VEGF (Reinke and Sorg, 2012).

Proliferation

In the proliferative phase, angiogenesis, collagen deposition, granulation tissue formation, and epithelialization occur (Behm et al., 2012). Fibroblasts are the key cells involved in production of extracellular matrix (Harding et al., 2002). The fibroblasts are stimulated by growth factors released from the platelets, including TGF-β, IGF-1 and PDGF (Teller and White, 2011). In the third day, the wound will become rich in fibroblasts which cause to precipitate extracellular matrix proteins (hyaluronan, fibronectins and proteoglycans) and then produce collagen and fibronectin. This results in fibrous tissue formation which along with vessels and macrophages replaces the clot at the site of the wound that is called granulation tissue. This is composed of a different types of collagen particularly type III collagen (Young and McNaught, 2011). In this phase of

wound healing, collagen type III is predominant, giving the feature of tensile strength to the newly created tissue (Olczyk et al., 2014). The matrix in and around the wound margin is degraded by different enzymes such as matrix metalloproteinase and plasminogen activators. The effect of matrix metalloproteinase is regulated by tissue inhibitors, which is important in wound healing by preventing excessive matrix degradation (Harding et al., 2002).

Angiogenesis

A massive angiogenesis supplys oxygen and nutrients necessary for the wound healing process (Rodero and Khosrotehrani, 2010). Angiogenesis begins 1 to 2 days after vessel disruption and can be evident within about 4 days after injury (Teller and White, 2011). In response to hypoxia, VEGF is released which in combination with the other cytokines, begin angiogenesis and repair the damaged vessels (Young and McNaught, 2011).

Epithelialization

Within several hours after injury, the process of epithelization is stimulated by growth factors such as EGF and TGF-α that are produced by activated wound macrophages, platelets and keratinocytes (Diegelmann and Evans, 2004). This process begins with epidermal thickening along wound edges (Teller and White, 2011). Keratinocytes from the wound edges migrate to the wound bed, between the wound dermis and the fibrin clot. This migration is facilitated by the production of specific proteases such as the collagenase produced by the epidermal cells to degrade the extracellular matrix (Rodero and Khosrotehrani, 2010). Epithelial cells continue to migrate and proliferate until they contact with epithelial cells coming from other directions (Teller and White, 2011).

Remodeling

The final phase of wound healing is remodeling and it may last for 1-2 years or more. In remodeling stage, fibroblasts differentiate into myofibroblasts. The first function of myofibroblasts is granulation tissue regeneration by producing new extracellular matrix, contraction and remodeling of wound and formation of scar tissue. Myofibroblasts differ from normal fibroblasts by the expression of alpha smooth muscle actin (α-SMA), which is most commonly used as marker for myofibroblasts characterization and it also convert them to contractile cells. They are also characterized by expression of the ED-A fibronectin and the enhanced synthesis of several ECM proteins and growth factors. Conversion of fibroblast to myofibroblast and expression of α-SMA depend on combination of mechanical tension and TGF-β1 (Eckes et al., 2010).

During wound maturation period, the content of the ECM undergoes specific changes. Produced type III collagen in proliferative phase are replaced by stronger type I collagen (Reinke and Sorg, 2012). Since type III collagen have no specific structure and doesn't provide enough strength, its destruction depends on matrix metalloproteinases and inhibitors, which they are produced by macrophages, keratinocytes and fibroblasts in response to cytokines, growth factors or cell contact with ECM. Type I collagen are in a parallel orientation and do not interlace like an intact dermis (Occleston et al., 2010; Reinke and Sorg, 2012). With the progress of the remodeling phase, the amount of fibroblasts decreases, the vascular density is lowered, and disabled cells are destroyed by apoptosis (Olczyk et al., 2014).

In chronic wounds, overexpression of proteolytic enzymes leads to destruction of ECM and essential growth factors. Moreover, neutrophils infiltration and proteinase–antiproteinase imbalance results to enhanced matrix destruction and chronic wounds with chronic inflammation (Behm et al., 2012).

Role of Mesenchymal Stem Cells in Wound Healing Process

Stem cells have the potential to differentiate into other cell lineages and the capacity of self-renewing. The presence of stem cells ensures tissue damage regeneration in different tissues and functional disorders improvement. These cells can be divided into two categories: embryonic stem cells and adult stem cells such as MSCs. The MSCs can play effective roles in injury improvement including the wound healing process (Wu et al., 2007). The development of therapies using stem cells in the wound healing has mainly relied on adult stem cells, especially mesenchymal stromal cells or mesenchymal stem cells (MSCs). These stem cells have self-renewing potential and capable of differentiating into various cells lineages (Chen et al., 2009). MSCs can migrate to sites of injury in response to chemotactic signals modulating inflammation, repairing damaged tissue, and facilitating tissue regeneration. These cells have pivotal roles in inflammation, proliferation and remodeling phases (Maxson et al., 2012).

MSCs are a category of stem cells originated from mesodermal germinal layer and they are formed only a very small percentage of cells in the bone marrow about 0.01-0.0001% of mononuclear cells (Yolanda et al., 2014). MSCs can also be isolated from different tissues, such as umbilical cord, endometrial polyps, bone marrow, and

adipose tissue (Ding et al., 2011). MSCs can differentiate into osteoblasts, chondrocytes, adipocytes and bone marrow stromal fibroblasts under the proper conditions. The stem cells retain high degree of flexibility and are capable of regeneration of skin progenitor cells including the keratinocyte stem cell (KSCs) (Fathke et al., 2004).
When tissue undergoes damage, bone marrow-derived stem cell migrate towards the wound site, because a small number of mesenchymal stem cells is always present in peripheral blood. Thus, in severe injury, the number of circulating stem cells increases to accomplish reconstruction properly (Chen et al., 2009).

Immunomodulation

Another mechanism of action of MSCs is that they directly weaken immune response in order to decrease inflammation. Indeed, they decrease secretion of the proinflammatory cytokines while increasing the production of anti-inflammatory cytokines. These anti-inflammatory property make them particularly useful to chronic wounds healing so that these cells promote the inflammatory phase into the next stage of healing (Yolanda et al., 2014).
In the inflammation phase, pre-inflammatory mediators, such as IFN-δ, TNF-α and IL-1β can activate regulatory functions in MSCs that enable them to modulate the immune response. In this phase, the MSCs can inhibit the employment, proliferation, and biological activity of mast cells, T cells, B cells, and natural killer cells (NK), thus, they weaken the severe immune response to injury (Jackson et al., 2012a). The inflammatory wound environment also stimulates cyc;ooxygenase 2 (COX2) activity in MSCs, which leads to upregulation of prostaglandin E2 (PGE2) and a change in wound function for desired regeneration of dermis (Jackson et al., 2012b). In addition to weakening T-cell proliferation, PGE2 can modify the behavior of leukocytes resident in the wound, corresponding to decreased expression of IL2 and INF-δ, and increased expression of IL4 and IL10 (Jackson et al., 2012a).
Indole- amine-2,3-dioxygenase *(*IDO) produced by MSCs suppresses many immune cells, such as T cells and NK cells. IDO is the first rate-limiting enzyme in the degradation of tryptophan through the kynurenine pathway leading to the tryptophan depletion. The reduction in local tryptophan concentration and the production of tryptophan metabolites, which cause immunomodulatory as well as the immunosuppressive effects of IDO-expressing cells (Hass and Otte, 2012; Shi et al., 2012).
These finding suggest that the immunomodulatory function of MSCs is very important for prevention of excess inflammation and wound healing (Murphy et al., 2013).

In addition to anti-inflammatory activity, MSCs have also antimicrobial effect. This mechanism is based on secretion of LL-37, a peptide with a broad array of antimicrobial properties including suppressing wide spectrum microbial defense via disruption of bacterial cell membranes (Nuschke, 2014).
Due to the activity of immune cells at the wound site, ROS produced by neutrophils, including superoxide, hydrogen peroxide and alkyl peroxides, are highly cytotoxic compounds that prepare the sterile environment for wound, but these ROS increase collagen deposition. ROS exposure for long time during wound healing results in enhanced fibrogenesis and accumulation of fibrotic tissues through a mechanism involving membrane lipid oxidation and induction of TGF-β1. Nitric oxide produced by MSCs in the wound can remove ROS and produce reactive nitrogen species such as peroxynitrite. Although products of these reaction can also be cytotoxic, they react more slowly than ROS and prevent oxidative damage to DNA and membrane lipids (Jackson et al., 2012b). MSCs also increase the nitric oxide synthesis significantly in response to the interaction with T-cells in the proinflammatory environment. Nitric oxide is complementary to prostaglandin E2 for the inhibition of T-cell through suppression of signal transducer and activator of transcription (STAT5) phosphorylation in T cells and induction of immune cell apoptosis (Jackson et al., 2012b; Shi et al., 2012).

Angiogenesis Promotion

Cell growth, proliferation, migration that mediate injured tissue healing require energy, which provide by blood capillary. Thus, blood vessel formation is the essential step in wound healing to protect granulation tissue and survive keratinocytes. Secretion of various factors involved in angiogenesis, such as VEGF, IGF-1, and angiopoietin-1 from MSCs is the powerful cues to promote proliferation, migration and differentiation of endothelial cells leading to increased angiogenesis. MSCs also express paracrine factors such as adrenomedullin to promote vascular stability and vasoprotection, which protect the healing process (Jackson et al., 2012b).
MSCs express stromal-derived factor-1 (SDF-1), VEGF and other important cytokines for angiogenesis, including PDGF-BB ،FGF ،Ang-1 ، IGF-1 ،MMP, IL-8 and IL-6. VEGF is a homodimer glycoprotein that stimulates the

recruitment and migration of endothelial cells, increasing angiogenesis. VEGF enhances Ang-1 expression, which acts via phosphorylation of Tie2 receptor. The Ang-1/Tie2 interaction could mediate maturation of neovessels into more complex and functional vasculature (Li et al., 2013; Zou et al., 2012). The SDF-1 activity is essential for endothelial cells survival and recruitment of stem cells to sites of injury. SDF-1α not only acts as signal to the progenitor cells with chemokine receptor CXCR4 recruitment to hypoxic tissue, but also it is a signal for retention of angiogenesis of the bone marrow-derived stem cells (Bollag and Hill, 2013).

Paracrine Signals

Paracrine signals can effect on adjacent cells; for example, VEGF not only supports neovascularization but also increases keratinocyte proliferation, which suggests a paracrine property for MSCs in wound re-epithelialization. MSCs produce various secretory factors, which play a role crucial in stimulation of skin fibroblast proliferation, angiogenesis, and collagen deposition. These cells respond to the local inflammation and hypoxic conditions of wound environment, and they protect important wound healing events, such as matrix deposition and blood vessel formation by stimulation of increasing in the rate of proliferation, differentiation, and growth factor production such as VEGF and FGF (Balaji et al., 2012). Many of these signaling mediator molecules have been studied (Table-1). MSC paracrine signaling regulates the cells responses at the wound site, which is the primary mechanism for the beneficial effects of MSCs on wounds, leading to reduce inflammation, stimulate angiogenesis, and induce cell migration and proliferation. Analyses of MSC-conditioned medium indicate that MSCs secrete many famous mediators that have role in healing process, including growth factors, cytokines, and chemokines, specifically VEGF, PDGF, bFGF, EGF, keratinocyte growth factor (KGF), and TGF-β (Maxson et al., 2012). MSCs also secrete mitogens such as TGF-α, TGF-β, HGF, EGF, FGF-2, and IGF-1, which promote proliferation of keratinocytes, dermal fibroblasts and endothelial cells (Jackson et al., 2012b; Murphy et al., 2013).

In the final stage of wound healing in adults, the unusual matrix deposition may be associated with the scar production. The growth factors regulation can control the scar formation. MSCs produce various cytokines and growth factors that have anti-fibrotic properties, including hepatocyte growth factor (HGF), IL-10 and adrenomedullin. HGF attenuates fibrosis and scar formation through a variety of mechanisms. In response to HGF, fibroblasts reduce the expression of TGF-β1, collagen type I and collagen type III. HGF also enhances the expression of MMP-1, MMP-3 and MMP-13 in fibroblasts, resulting change the ECM. HGF also prevents the fibroblast differentiation to myofibroblast, thereby restricting pro-fibrotic function of these cells (Jackson et al., 2012b).

IL-10 has direct effects on fibrosis through down-regulation of the expression of TGF-β1 in macrophages and T-cells, and reprogramming wound fibroblasts to regeneration of ECM by up-regulation the expression of metalloproteinases and down-regulation the expression of collagen (Li and Fu, 2012). IL-10 also prevents excessive collagen deposition by decreasing the expression of proinflammatory cytokines in the wound, such as IL-6 and IL-8 (Jackson et al., 2012b; Nuschke, 2014).

Cell Therapy and Methods of Stem Cell Transplantation to Wound Site

Cell therapy can be defined as a set of strategies to use live cells with therapeutic purposes. In this method, the cells are multiplied through *in vitro* cell proliferation in the desired volume and are provided to the target tissue. The aim of the therapy is to repair, replace or restore the biological function of a damaged tissue or organ (Yolanda et al., 2014). The use of stem cells is a new hope for chronic wound healing. Since transplant rejection is one of the problems in cell therapy, it is one of the important benefits of MSCs that allogeneic MSCs promote low immune reactions in hosts after transplantation. In addition, MSCs express the major levels of histocompatibility complex (MHC) class I but does not express MHC class II or molecules CD80, CD86, or CD40, which are involved in controlling humoral or cell-mediated immune responses. Therefore, MSCs have low inherent immunogenicity and contain an immunomodulation and immunosuppression function, which makes them appropriate candidate for autologous and allogeneic transplantation (Volk, 2010). Beside, bone marrow-derived MSCs synthesize higher amounts of various growth and angiogenic factors compared to native dermal fibroblasts, indicating a potential use in accelerating wound healing.

Today with technological advances, MSCs can be isolated from the patient's bone marrow and other tissues such as adipose tissue, nerve tissue, umbilical cord blood, and dermis (Chen et al., 2009).

Table 1. Growth factors released from mesenchymal stem cells during wound healing.

Abbreviation	Growth Factor	Target cell	Functions
EGF	Epidermal growth factor	Endothelial cells	Angiogenesis (Li and Fu, 2012)
PGE2	Prostaglandin E2	Leukocytes	Modulators of inflammation (Jackson et al., 2012a)
IDO	Indoleamine 2,3-dioxygenase	Leukocytes	Suppression of inflammation (Shi et al., 2012)
PDGF	Platelet-derived growth factor	Endothelial cells	Angiogenesis, Endothelial cell proliferation (Li and Fu, 2012; Volk, 2010)
VEGF	Vascular endothelial growth factor	Endothelial cells	Angiogenesis, Vascular permeability (Li and Fu, 2012; Zou et al., 2012)
HGF	Hepatocyte growth factor	Fibroblasts, Endothelial cells	Angiogenesis(Li and Fu, 2012), anti-fibrotic, inhibition of myofibroblast differentiation (Jackson et al., 2012a)
SDF-1	Stromal cell-derived factor 1	Endothelial cells	Angiogenesis (Zou et al., 2012)
IL-10	Interleukin 10	Leukocytes, Fibroblasts	Modulators inflammation, anti-fibrotic, inhibition of myofibroblast differentiation (Jackson et al., 2012a)
AM	Adrenomedullin	Fibroblasts, Endothelial cells	vascular stability, vasoprotection , anti-fibrotic (Jackson et al., 2012b)
Ang-1	Angiopoietin 1	Endothelial cells	Angiogenesis (Zou et al., 2012)

It seems that using a fibrin polymer spray that contains autologous bone marrow-derived or adipose tissue-derived MSCs is proper way for topical delivery. This procedure concentrates the cells and provides a non-toxic matrix from which cells migrate into wound beds (Shi et al., 2013). Fibrin spray supports junction, proliferation, and migration of MSCs. Fibrin in spray increases the viability of MSCs (Sorrell and Caplan, 2010). This approach is used to accelerate the rate of healing of acute and non-healing cutaneous wounds in both humans and mice. In a study, an autologous graft composed of autologous skin fibroblasts on biodegradable collagen membranes combined with autologous MSCs applied directly to the wound, which leads to a decrease in the wound size, and an increase in the vascularity of the dermis and dermal thickness of the wound (Chen et al., 2009).

Cell Therapy Limitations for Skin Wound Healing

Delivering stem cells to the wound is a technical challenge. In order to optimize the therapeutic potential of MSCs, the delivery medium should support cell adhesion, proliferation, migration, and differentiation. The unfavorable non-healing wound environment, characterized by increased proteolytic activity and chronic inflammation, and it is another challenges to cell viability after delivery (Falanga et al., 2007). One potential limitation to use of MSCs for treating chronic wounds is varying degrees of cell survival after implantation, which might shorten the therapeutic effects in the long-term. Another limitation to using MSCs as a standard therapy in any context is a general functional heterogeneity that turn standardization of these cells for manufacturing and quality control purposes to a severe challenge (Nuschke, 2014).

Moreover, the use of MSCs as a therapeutic agent needs to *ex vivo* expansion that could remain as a problem, because heterogeneity may limit the normal lifespan (Brower et al., 2011). This treatment approach generally requires to the MSCs that must be cultured in sufficient numbers for topical application; this may not be an important issue for small chronic wounds, but is impractical for large wounds treatment. The severe burns and trauma that lead to bone marrow damage decrease the MSCs as a result of silver sulphadiazine toxicity used for treatment of burn wound infection. The bone marrow MSCs also significantly decrease with age that may decrease the applicability of using autologous MSCs for chronic wounds (Chen et al., 2009). In total, according to the potential of these cells to control of inflammation and wound reconstruction, with further development in culture and transplantation techniques we hope to use the cells as an excellent strategy for wound healing,

particularly in chronic and extensive wounds.

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Research Article

Investigation of Melanogenic Factors Gene Expression in Human Adult and Neonate Retinal Pigment Epithelium Cell Cultures

Azam Mohammadian [1*], Zahra-Soheila Soheili [2], Razieh Jalal [1], Abouzar Bagheri [3], Shahram Samiei [4], Hamid Ahmadieh [5]

[1] *Department of chemistry, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *National Institutes of Genetic Engineering and Biotechnology, Tehran, Iran*
[3] *Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran*
[4] *Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine,Tehran, Iran*
[5] *Ophthalmic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

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Abstract

Retinal pigment epithelium is responsible for maintaining the structural integrity of the retina by an efficient defense against free radicals, photo-oxidative exposure and light energy. For this purpose the main RPE line of defense is melanosomes. Melanin content of retinal pigment epithelium cells in adults and neonates reveals remarkable variations. In the current study we compared melanogenic factors gene expression levels in human adult and neonate RPE cells in culture. RPE cells from adult and neonate human eye globes were isolated and then cultured in DMEM: F12 (1:1) containing 10% FBS. Expression of RPE65 and Cytokeratin 8/18 markers in isolated cells was confirmed by immunocytochemistry. To evaluate the responsible factors in the pathway of melanin biosynthesis, gene expression of orthodenticle homeobox 2, microphthalmia-associated transcription factor A and H as prominent transcription factors, tyrosinase and dopachrome tautomerase as effective enzymes in the melanin biosynthetic pathway were examined in human neonate derived RPE cells compared to human adult derived RPE cells in culture. According to the Real-Time RT-PCR data, gene expression of MITF-H, OTX2, TYR and DCT in RPE cells of the neonates showed a significant increase compared to the adults. With increasing passage number, gene expression of MITF-H, OTX2, TYR and DCT showed remarkable decline. According to the role of OTX2 and MITF-H as the main transcription factor effectors on the TYR and DCT, restoration of OTX2 and MITF-H gene expression may be retain melanin content in RPE cells.

***Keywords*:** Human RPE cells, Melanogenic transcription factors, Enzymes

Introduction

Retinal pigment epithelium (RPE) is a single layer of post-mitotic pigmented cells; RPE layer has key roles in the pathophysiology of several diseases of the eye and vision (Cai et al., 2009; Nussenblatt and Ferris III, 2007; Pfeffer and Philp, 2014). A prominent distinction characteristic of RPE cells is the pigment melanin. Melanin plays an important role in protecting the retina by absorbing radiation and scavenging free radicals and reactive oxygen species. Melanogenesis, is an enzymatic process catalyzed by tyrosinase (TYR), TYR-related protein 1 (Tyrp1) and dopachrome tautomerase (DCT) that convert tyrosine to melanin pigments. It is presumed that RPE melanogenesis is just prenatally, since TYR was detected only in human early stage embryos and was not detectable after gestation stopped (Schraermeyer et al., 2006; Boulton, 2014; Pfeffer and Philp, 2014). With age melanin content of RPE cells and its protective properties reduced and resulting in risk of degenerative retinal diseases such as AMD and RP (Boulton 2014; Le et al., 2014; Zarbin et al., 2014). Differentiated RPE cells do not divide and remain functional throughout the life of an individual. However cultured human RPE cells can be grown in large quantities and used in biochemical and functional assays or transplantation studies (Maminishkis et al., 2006; Klimanskaya et al., 2008; Hu and Bok, 2001). Nevertheless, the value of cultured RPE cells depends on its ability to retain functional and genetic characteristics of the native tissue (Strunnikova et al., 2010). Retinal pigment epithelium cell lines do not show features typical of a functional RPE, such as pigmentation and expression of specific markers (Aruta et al., 2011) and deficiency of an *in vitro* system recapitulating all the features of *in vivo* RPE cells has been one of

Corresponding authors E-mail:
* soheili@nigeb.ac.ir

the limitations in molecular and functional studies of the RPE. Orthodenticle homeobox 2 (OTX2) is a transcription factor expressed not only at the earliest stage of the eye morphogenesis (Simeone et al., 1992) but also in postnatal RPE and adult eye (Baas et al., 2000). Otx2 controls the expression of several groups of genes involved in RPE-specific functions, especially melanogenesis, pH regulation, retinol metabolism, and metal concentration (Takeda et al., 2003; Housset et al., 2013). Microphthalmia-associated transcription factor (MITF) consists of many isoforms with different N-termini. A, H and D are the main isoforms of this gene in RPE cells (Bharti et al., 2008; Capowski et al., 2014). Many transcriptional targets of MITF suggest its involvement in multiple aspects of melanogenesis (Levy et al., 2006) by regulating the expression of TYR and DCT (Cheli et al., 2010; Ludwig et al., 2004; Raviv et al. 2014). Moreover MITF has been linked to control of gene expressions involved in survival, cell cycle control, and cell morphology (Johnson et al., 2011). Because of the principal impression of MITF-A, MITF-H, OTX2, TYR and DCT in melanin biosynthesis and the key roles of RPE cells in visual function, they have been evaluated in the current study to distinguish their differential gene expression in different cell culture passages and between adult and neonate human RPE cells.

Materials and Methods

Cell Culture and Sample Preparation

RPE cells were isolated from neonatal and adult human cadaver globes provided by central eye bank of Iran within 24 hours of death and cultured in DMEM: F12 (1:1) supplemented with 10% FBS. After cultures reached to 80-90% confluency, the cells were passaged. RPE cells' RNA was extracted and was reverse transcribed using cDNA synthesis kit and subjected to amplification by Real-Time PCR.

Cell Identification

Cell cultures were evaluated by means of morphology and expression of characteristic molecular markers. All the cultures were considered under light microscope in terms of morphology. Then RPE cells were cultured on coverslips in 24-well plate. Coverslips were fixed by -10°C methanol for 10 minutes. The cells were made permeable using Teriton x-100 (0.25%) and cultures were blocked in 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature. Specific mouse anti-human cytokeratine 8/18 monoclonal antibody (1:1000; Santa Cruz, USA) for epithelial characteristics confirmation of isolated cells and rabbit anti-human RPE65 polyclonal antibody (1:100; Santa Cruz, USA) against the specific protein of RPE microsomal membranes were utilized to confirm the RPE characteristics of isolated cells. Fluorescein isothiocyanate (FITC) conjugated antibodies (goat anti-mouse IgG, goat anti-rabbit IgG, Santa Cruz, USA) against the above-mentioned antibodies were diluted (1:400) and used to detect immunoreactivity of cultures to primary antibodies. After final washing, the slides were incubated with 4, 6-diamidino-2-phenyindole dihydrochloride (DAPI) (1.5 mg/ml, Santa Cruz, USA) for 10 minutes in order to stain nuclear DNA. The slides were examined by Axiophot Zeiss fluorescence microscope (Zeiss, Germany) equipped with a 460 nm filter for DAPI dye and a 520 nm filter for FITC conjugated antibodies.

Real-time RT-PCR

Total RNA was extracted from RPE cells in each examined passage (between the first and the 7th passages) by RNA extraction kit (Qiagen, Germany). Concentration and purity of the isolated RNA were determined using spectrophotometric analysis and the integrity of the RNA was verified by means of electrophoresis in an agarose gel followed by ethidium bromide staining. Reverse transcription reaction was performed with oligo dT primers and a superscript reverse transcriptase kit (Promega, USA). Then quantitative real-time RT-PCR was performed by a SYBR Green QPCR master mix (Roche, USA). PCR parameters were initial denaturation (one cycle at 95°C for 10 minutes); denaturation, amplification and quantification for 40 cycles at 95°C for 30 seconds, 52-60°C for 17 seconds, and 72°C for 25 seconds; melting curve, 65°C, with the temperature gradually increased (0.5°C) to 95°C. mRNA expression was normalized to the levels of GAPDH mRNA, and the changes were calculated according to standard curve and efficiency (E) for each primer. Sequences of primers used for real-time PCR were shown in table 1.

Statistical Analysis

3 neonate globs and 2 adult globs were included in this study. The real-time RT-PCR analysis was performed at least in 3 independent experiments. Each sample was run and examined in duplicate. Differences between groups (different passages in neonates and adults) were analyzed using the t-test. $P < 0.05$ was considered statistically significant.

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Table 1. Primer Sequences

Sequence definition	Sense primer	Anti-sense primer
MITF-A	AAGTCGGGGAGGAGTTTCAT	CGTAGCAAGATGCGTGATGT
MITF-H	TTCAGATGTTCATGCCATGCTC	GCGTAGCAAGATGCGTGATG
OTX2	QT00213129	
TYR	QT00080815	
DCT	QT00033523	
GAPDH	QT01192646	

*Sequence of primers for MITF-A and MITF-H have been shown.
For OTX2, TYR, DCT and GAPDH predisigned primers were bought with the presented cat. numbers.

Results

Isolated Cells Exhibited Specific Markers of RPE Cells

In terms of morphology, confluent monolayers of RPE cell cultures exhibited epithelial morphology and heavy pigmentation (Figure. 1). ICC revealed that the isolated cells expressed both cytokeratin 8/18 and RPE65, confirming their identity as RPE cells (Figure. 2).

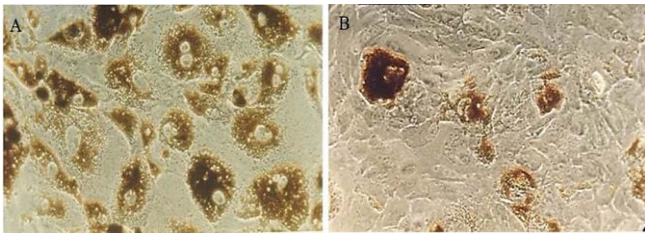

Figure 1. Human cultured RPE cells grew as a monolayer of cuboidal epithelial cells arranged in a regular hexagonal pattern (magnification: (A) 10x40, (B) 10x20).

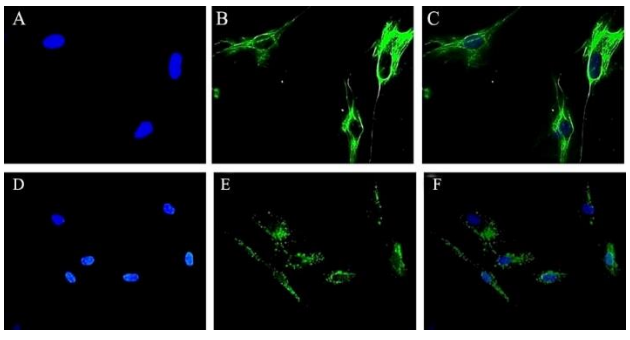

Figure 2. Human cultured RPE cells subjected to ICC with cytokeratin 8/18 and RPE65 antibodies. (A) Nuclei stained blue with DAPI. (B) The RPE cells stained positively for the FITC-conjugated cytokeratin 8/18 antibody (green). (C) Merged image (FITC-labeled cytokeratin 8/18 and DAPI) (magnification: 10x40). (D) DAPI-stained RPE cell nuclei (blue). (E) The RPE cells stained positively for the RPE65 antibody (green). (F) Merged image (FITC-labeled RPE65 and DAPI) (magnification: 10x20).

Neonate RPE Cells Revealed Different Patterns of Melanin Biosynthesis Responsible Factors

MITF-A gene expression did not alter at different passages of neonate samples (Figure. 3A). MITF-H decreased at passage 7 in neonate RPE cells when compared to, specially, passage 1 (Figure. 3B). OTX2 significantly was decreased in passages 4-7 of neonate samples compared to the other passages and specially passage 1 and 2 (Figure. 3C). TYR gene expression decreased at passages 4-7 in neonate samples compared to the other passages and specially passage 1 (Figure. 3D).

DCT was remarkably decreased at passages 2-7 compared to the passage 1 of neonate samples (Figure. 3E).

Adult RPE Cells did not Show Significant Variance of Melanogenic Factors Gene Expressions

MITF-A (Figure. 4A), MITF-H (Figure. 4B), OTX2 (Figure. 4C), TYR (Figure. 4D) and DCT (Figure. 4E) gene expression did not alter at different passages of adult samples.

Cultured Neonate and Adult RPE Cells Manifest Significant Variance of Melanogenic Factors

Neonate gene expression of MITF-A at different passages was not significantly different in adults RPE cell cultures (Figure. 5A).

MITF-H (Figure. 5B), OTX2 (Figure. 5C) and TYR (Figure. 5D) gene expression were significantly increased at passages 1, 2 and 3 of neonates compared to the adults.

DCT remarkably increased at passages 1 and 2 of neonates compared to the adults RPE cultures (Figure. 5E).

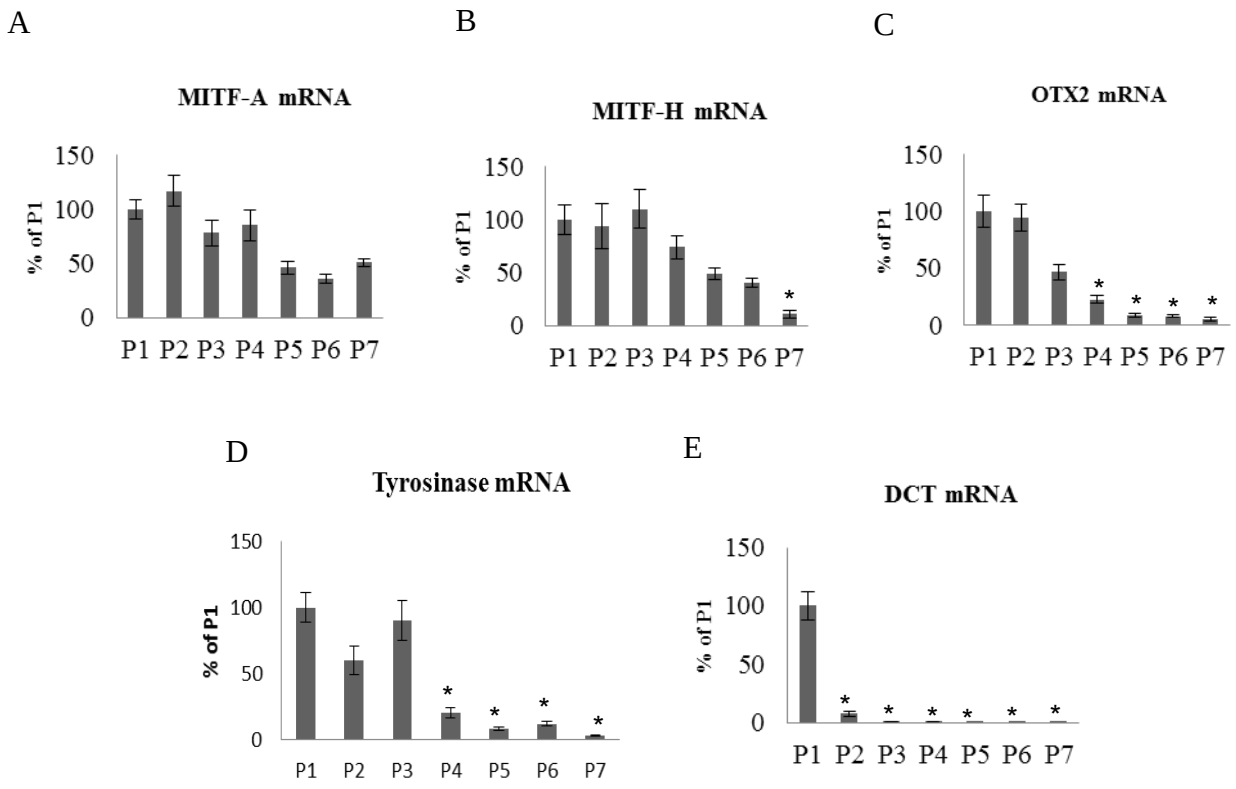

Figure 3. Quantitative real-time RT-PCR analysis of MITF-A, MITF-H, OTX2, TYR and DCT in passages 1-7 of cultured neonate RPE cells. RPE cell preparation and RNA extraction were performed as described in methods. Relative gene expression was determined by quantitative real-time PCR. mRNA levels were normalized to GAPDH and presented as percentages of control values. (B) MITF-H at passage 7, (C) OTX2 at passages 4-7, (D) TYR at passages 4-7 and (E) DCT at passages 2-7 decreased compared to the other passages specially regarding passage 1. *P <0.05

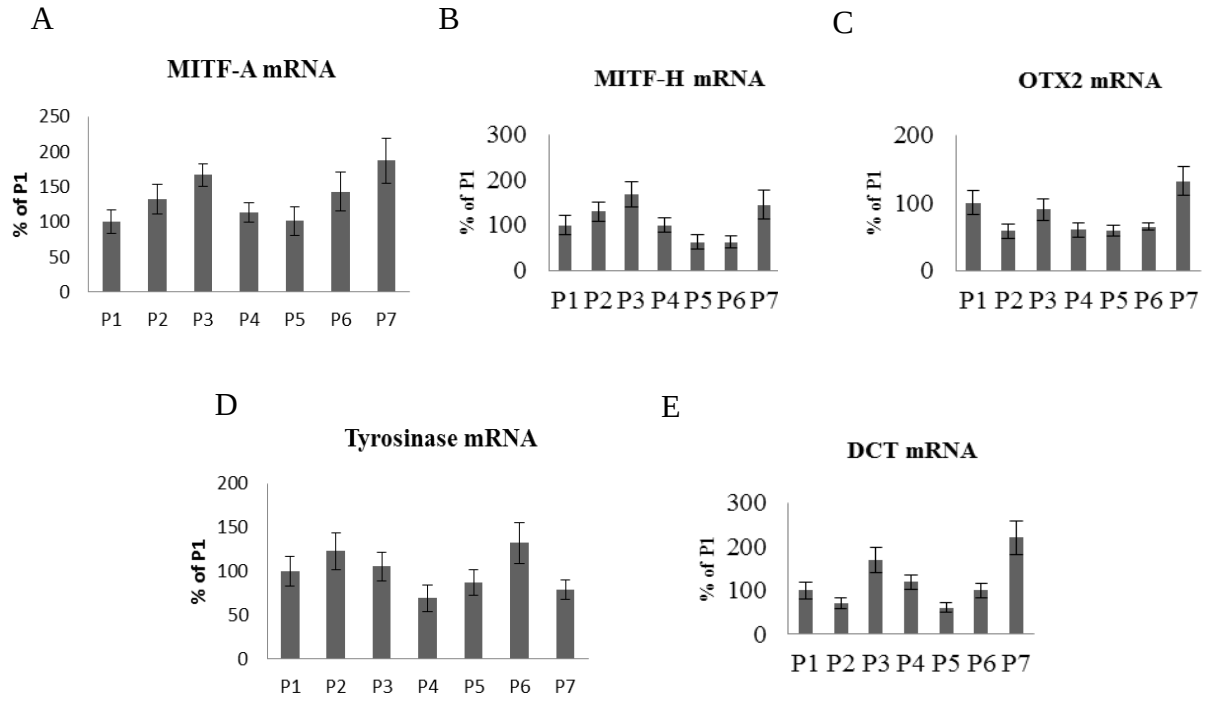

Figure 4. Quantitative real-time RT-PCR analysis of MITF-A, MITF-H, OTX2, TYR and DCT in passages 1-7 of cultured adult RPE cells**.** RPE cell preparation and RNA extraction were performed as described in methods. Relative gene expression was determined by quantitative real-time PCR. mRNA levels were normalized to GAPDH and presented as percentages of control values. Gene expression of (**A**) MITF-A, (**B**) MITF-H, (**C**) OTX2, (**D**) TYR and (**E**) DCT did not alter in different passages of adult samples. *P <0.05.

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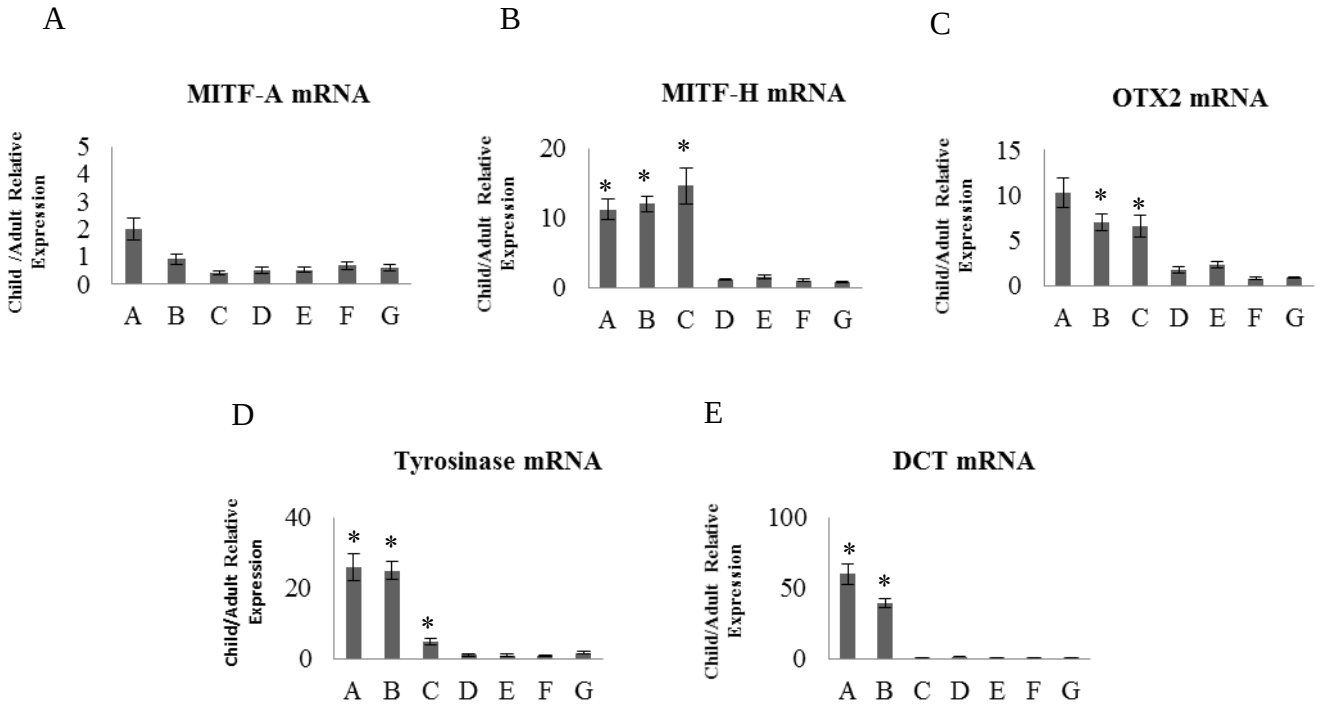

Figure 5. Comparison of MITF-A, MITF-H, OTX2, TYR and DCT gene expression in passages 1-7 of cultured neonate RPE cells compared to adult RPE cells. (B) MITF-H, (**C**) OTX2 and (D) TYR gene expression were significantly increased at passages 1, 2 and 3 of neonates compared to the adult samples, while (E) DCT remarkably increased at passages 1 and 2 of neonates compared to the adult samples. *P <0.05.

Discussion

In the present study we declared that gene expression of MITF-H in higher passages of neonate samples significantly decreased and subsequently gene expression of TYR and specially DCT enzymes was reduced. As a result, overexpression of MITF-H in RPE cell cultures could be a solution to retain the features of RPE in its *in vivo* circumstance and growing native RPE cells in large quantities for studying their biology, biochemical features and functional properties.

OTX2 is another transcription factor which plays important functions in the melanin synthesis. OTX2 was identified in nucleus of RPE cells. It incollaboration with MITF, or alone, regulates expression of principal RPE cell genes. Overexpression of OTX2 in neural retina induces pigmentation phenotype, this factor binds to the promoter of TYR and DCT and activates their expression (Takeda et al., 2003; Martínez-Morales et al., 2003; Reinisalo et al., 2012).

Our results showed gene expression of OTX2 significantly decreased at earlier passages of neonate samples and this decrease was prominently more than MITF-A and MITF-H. Gene expression of TYR and DCT decreased at early passages too. Expression of TYR and DCT is presumably primarily under regulation of OTX2 more than two other aforementioned transcription factors.

Human RPE cells contain a large amount of melanin, which is produced during the prenatal period but polymerization of melanin continues to occur in melanosomes until turning to approximately two years old, when the RPE contains only mature melanosomes (Boulton and Dayhaw-Barker, 2001; Boulton, 2014).

No melanin production could be demonstrated *in vitro* and *in vivo* status of RPE cells after birth. Humans have a substantial amount of melanin in their RPE cells. Melanin content of RPE cells decreases with increasing age and sharply reduces their protective working and may be responsible for the occurrence of various retinal diseases like AMD, the leading cause of irreversible blindness among the elderly in industrialized nations (Panda-Jonas et al., 1996). Melanin content in cultured RPE cells also decreased rapidly pursuant to cell division. In normal culture conditions, RPE cells lose many traits that are important for the proper

functioning of vision, including bearing melanin. Melanin content of cultured RPE cells decreases with increasing passage number, so that the cells in the higher passages have a little melanin content (Lu et al., 2007; Pfeffer and Philp, 2014).

Some reports showed that adult human RPE cells cultured with Ca^{++}-switch method (low Ca^{++}) expressed TYR and produced melanin (Rak et al., 2006). Expression of various genes in RPE cells, including TYR and its enzymatic activity are induced in cultured human adult RPE by phagocytosis of ROS (Julien et al., 2007; Chowers et al., 2004).

Our Results showed among MITF-A, MITF-H and OTX2 transcription factors, gene expression of MITF-H and OTX2 was significantly dissimilar between different passages of neonate and adult RPE cells in culture.

For researches who study RPE cells biology MITF have been the center of attention because it is involved not only in the induction of pigmentation but also in regulating cell proliferation, structure of the cytoskeleton and cell differentiation. It is called as one of the key elements in RPE cell behaviors (Tsukiji et al., 2009). MITF affects gene expression of DCT and TYR enzymes positively and its reduction will negatively affect gene expression of mentioned enzymes and therefore melanin content (Yasumoto et al., 1997).

RPE cells display prominent role in defense against free radicals and photo-oxidative exposure and light energy. Melanosomes are the key players in RPE cells preservative role. Our data showed variation of OTX2 and MITF-H transcription factors gene expression especially OTX2 is similar to melanogenic enzymes gene expression and proposed restoration of MITF-H and especially OTX2 gene expression in adult RPE cells may culminate to retain melanin content of the cells by recovering key enzymes in melanin synthesis pathway, e.g. TYR and DCT. Quantitative studies on the expression of various transcription factors and enzymes, relevant to the melanogenesis, combined to the rate of melanin production, may provide the *in vitro* models for studying melanogenesis of human RPE cells and could be used in experiments/purposes that require natively pigmented RPE cells.

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Research Article

Characterization and Molecular Identification of Extracellular Polymeric Substance (EPS) Producing Bacteria from Activated Sludge

Bahar Shahnavaz [1,3*], Simin Maroof [1], Mohsen Karrabi [2], Mansour Mashreghi [1]

[1] *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Department of Civil Engineering, Faculty of Engineering, Ferdowsi University of Mashhad, Mashhad, Iran*
[3] *Institute of Applied Zoology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*

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Abstract

The aim of this study was identification and characterization of highly efficient Extracellular Polymeric Substance (EPS) producing bacteria in activated sludge. Among 74 isolated bacteria, 20 EPS-producing bacteria were obtained from wastewater treatment plant of Bojnourd. EPS extraction was performed using absolute ethanol after glucose-enriched culture. Dry weight, concentration, and the ratio of carbohydrate to protein for the EPS of each isolate was then determined. Molecular identification of four bacteria with the highest EPS production was carried out using 16S rRNA gene amplification. The results showed that these bacteria are belonging to the genus *Bacillus*, *Pseudomonas* and *Klebsiella*. EPS productions were studied in different conditions (carbon and nitrogen sources, different levels of glucose and yeast extract and temperature) for the genus *Bacillus* and *Pseudomonas*.The production of EPS was observed highest while in the presence of 2.5 to 3% glucose and 0.5% yeast extract at temperature of 30 to 37°C.

***Keywords*:** EPS-producing bacteria, Sludge, 16S rRNA gene

Introduction

Many bacteria are able to excrete extracellular polymeric substances (EPS) outside of their cell walls (Vu et al., 2009). EPS exists in two forms, either attached to the cell (capsule) or as extracellular secretion (slime) (Hirst et al., 2003). EPS produced by microorganisms have attracted the attention of many researchers due to their versatile applications and various advantages. They play an important role in biotechnology such as textiles, pharmaceuticals, food, oil recovery and wastewater treatment processes (Maugeri et al., 2002; Zhang et al., 2003; Parikh et al., 2006; Yuksekdag et al., 2008; Patel et al., 2010). Nichols et al., (2005) reported that the extreme environmental conditions affect the bacterial behavior and consequently the production of EPS. It seems that EPS protect bacteria from environmental stresses. Studies have shown that some environmental conditions such as pH, temperature (Pengfu et al., 2001; Singh et al., 2011) and nutrient source (Mata et al., 2006; Pawar et al., 2013) can influence the EPSs production. EPSs are composed of carbohydrates, proteins, lipids, nucleic acids, glycoproteins and phospholipids (Lazarova et al., 1995; Czaczyk and Myszka, 2007). In recent years, EPS producing bacteria were isolated and identified from different habitats such as saline water (Llamas et al., 2010; Mata et al., 2006), soil (Pawar et al., 2013; Razack et al., 2013), food (Gamar- Nourani et al., 1998) and petroleum contaminated soil (crude petroleum oil) (Zaki et al., 2011). Identification of these bacteria using culture-dependent and molecular-based techniques indicates that they belong to different taxonomic groups (Vu et al., 2009). Identification of EPS producing bacteria in activated sludge helps not only to have better insight into the physico-chemical properties of sludge, but allows predicting its related processes such as sludge settling and dewatering. A fail flocculation of bacteria may occur due to insufficient EPS in activated sludge composition. EPS constituents are different depending on the type of microorganisms, microbial biofilms age and conditions of EPS-formation. These conditions include the amount of oxygen, nitrogen content, cell density, and environmental parameters (Vu et al., 2009).

Corresponding authors E-mail:
* *shahnavaz@um.ac.ir*

Activated sludge is a complex consortia of microorganisms formed in aeration conditions and responsible to remove the organic material from wastewater. In general, bacterial extracellular polymeric substances improve the formation of bioflocs in activated sludge and contribute to its structural, surface charge and settling properties (Houghton et al., 2001). Therefore, EPS produced by microorganisms affect sludge characteristics and consequently the efficiency of flocculation processes in wastewater treatment plants (More et al., 2014). Sheng et al., (2010) described that EPS secreted by bacteria play an important role in sludge bioflocculation because all active sites of EPS are free and able to form floc with biomass. In this study, the objectives were: (i) characterization and identification of EPS producing bacterial strains from municipal wastewater sludge and (ii) evaluation of different conditions in EPS production.

Materials and Methods

Site Sampling

An initial sample was taken from activated sludge in municipal wastewater treatment plant of Bojnourd. 50 ml of activated sludge was added in to 450 ml TSB (tryptic soy broth) medium, then incubated at 30ºC for 30 min at 150 rpm. After serial dilution, 100 µl of each dilution were cultured on TSB containing agar. In order to have maximum isolation, the culture plates were incubated at 22°C and 30°C for 24-48 h. The EPS-producing bacteria were selected based on their viscous and mucoid characteristics that enable colonies to have a string formation. The seed culture was produced in 20 ml TSB medium. 200 µl of standard bacterial suspension (1.5×10^8 CFU/ml) was added to the culture medium and incubated at 150 rpm and 30°C for 48 h. After getting a logarithmic growth phase, the isolates were inoculated in glucose medium (25 g/l glucose, 0.2 g/l $MgSO_4$-$7H_2O$, 2 g/l K_2HPO_4, 1 g/l KH_2PO_4, 1 g/l NH_4Cl and 0.05 g/l yeast extract). The initial pH of media was adjusted to 7.0. Glucose and $MgSO_4$ were sterilized separately and mixed aseptically with other ingredients before inoculation. In order to achieve sufficient viscosity, all samples were incubated in a shaker at 150 rpm and 30°C for 72 h (Subramanian et al., 2010).

EPS extraction

The glucose medium at the end of 72 h, became highly viscous. In order to extract the EPS, the culture medium was centrifuged at the speed of 6000 *g* at 4°C for 10 min to remove bacterial cells. The supernatant phase mixed with 2.2 volumes of absolute ethanol and then incubated at -20°C for 2 h. To collect the precipitated EPS, the mixture was centrifuged at 6000 *g* and 4°C for 15 min. The supernatant was removed and the pellet containing slime EPS was dried at room temperature in a laminar hood for 6 h (Subramanian et al., 2010). The dry weight of the extracted EPS was measured (APHA, 2005).

EPS Analysis

The total amount of protein and carbohydrate in EPS was quantified using the Bradford method (Bradford, 1976) and phenol-sulfuric acid method (Dubois et al., 1956) respectively. A standard curve of bovine serum albumin (BSA) was applied to calculate the amount of protein in each sample.

Bacterial Growth

EPS are referred to secondary metabolite produced in the late logarithmic phase or at the early stationary phase of growth. To evaluate the EPS production rate, 20 ml TSB medium was prepared in a 100 ml flask. Approximately 1.5×10^8 CFU/ml was added into the medium. The growth rate of bacteria was determined by the measurement of optical density of bacterial suspensions at 600 nm using spectrophotometer.

Effect of Different Carbon and Nitrogen Sources

To determine the best source of carbon for the production of EPS, various carbon sources such as glucose, fructose, sucrose, lactose and non-carbon sources were investigated by adding 1% (w/v) of each one to the production medium. 10% of each mentioned carbon sources were added to the culture medium. After inoculation, the samples were incubated at 30°C and 150 rpm for 24 h. After sufficient time, the EPS of desired strains were extracted. To determine the effect of glucose level in EPS production, glucose was used at 1, 1.5, 2, 2.5 and 3% (w/v) concentrations. Then, the effect of nitrogen sources (peptone, urea, ammonium nitrate, yeast extract and nitrogen-free medium) was evaluated by adding 0.1% (w/v) of each one to the production medium. To examine the effect of yeast extract level as the best nitrogen source, yeast extract was used at 0.005, 0.1, 0.3, 0.5, 0.7 and 0.9 % (w/v) concentration. To evaluate the effect of temperature, flasks containing inoculated production media were incubated at 20, 30, and 37°C. Culture conditions and extraction is similar to the previous step.

Bioflocculation Activity

Flocculation activities influenced by different extracted EPS, were calculated and measured using a modified method (Yun and Park 2003). A mixture of 5 g/l powdered activated carbon suspension with 0.1 ml extracted EPS in the presence of $CaCl_2$ (100 mg/l, as coagulant) was stirred with rapid mixing at 230 rpm for 2 min. A suspension without any bioflocculant addition, was used as a control under similar conditions. The suspension was then allowed to flocculate for 10 min and the supernatant of settled activated carbon suspension was withdrawn to measure the absorbance using UV spectrophotometer at 550 nm. The flocculation activity was calculated according to the following equation:

$$Flocculation\ activity\ (\%) = \left[\frac{A-B}{A}\right] \times 100$$

Where A and B are the supernatant optical density of the control and sample respectively, at 550 nm.

Biochemical and Molecular Identification of Bacteria

In order to identify the selected strains, gram staining, capsule staining, motility test, catalase activity, citrate utilization, H_2S production, indole production, and sugar fermentation (glucose, lactose, and mannitol) were performed. DNA genome analysis was conducted using the FastDNA® SPIN Kit. Genomic DNA of selected bacteria with high EPS production was extracted from fresh culture according to the manufacturer's instructions. The amplification of 16S rRNA gene was performed using universal primers (27F and 1492R). PCR was performed using 1.5 mM $MgCl_2$, 30 mM KCl, 10 mM Tris-HCl, 2.5 mM of each dNTP, 5–10 pmol of each primer, and 1U of *Taq* polymerase. PCR was carried out as follows: initial denaturation at 95°C for 2 min, 30 denaturation cycles at 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s, and final elongation at 72°C for 7 min. The sequencing was carried out at Macrogen, South Korea.

Phylogenetic Analysis and Comparison of Sequences

The partial 16S rRNA sequences of the isolates were compared with the NCBI and Ez-taxon databases. The GenBank accessions of the sequences are KR185341 to KR185344. Multiple sequence alignments were performed using online tools SINA (http://www.arb-silva.de/aligner/). The phylogenetic dendrograms were constructed by the Maximum likelihood method, and phylogenetic tree was evaluated by performing bootstrap analysis of 1000 data sets using MEGA (Tamura et al., 2011).

Statistical analysis

One-way analysis of variance and Tukey HSD test were done by Tukey's test with the R software (The R Development Core Team, 2007).

Results and Discusion

Screening and Isolation of EPS Producing Bacteria

A number of EPS producing bacteria have been isolated and characterized in last few years and protocols have been presented in literature (Subramanian et al., 2010; Zaki et al., 2011; Pawar et al., 2013). In this study, we aimed to isolate EPS producing bacteria from the slimy colony formation on the growth medium. Out of total seventy four isolated strains from mucoid colonies, 42 (56%) were gram-positive and the remaining 32 (44%) were gram-negative having morphology ratio as 45 rods and 29 cocci shaped strains. Among these 74 isolates, 20 strains having the highest appearance of mucoid colonies were selected and the EPS of each isolate extracted using ethanol. The EPS dry weight and EPS concentration varies from 0.06 to 0.46 g and 2.2 to 15.7 g/l, respectively (Table 1). The EPS concentration from bacterial strains of municipal wastewater sludge was reported as 4-35 g/l by Subramanian et al., in 2010. Right after, Razack et al., (2013) also showed that EPS concentration from *Bacillus subtilis* varied from 3.5 to 5.5 g/l. It was also discussed that variation in EPS concentration depends upon the environmental parameters and extraction method (Metzger et al., 2009; Orr et al., 2009). Isolate BS2 in gram-positive bacteria and BS8, BS9 and BS20 in gram-negative bacteria produce the highest EPS concentration, and have selected for future studies.

Chemical Composition of EPS

Total carbohydrate and total protein was extracted and evaluated from EPS using phenol-sulfuric acid and Bradford method (Table 1). The carbohydrate/protein (TC/TP) ratio measured in all extracted EPS samples varied from 1.42 to 15. This result is similar to the previous investigations which exhibit that carbohydrate was usually dominated in EPS composition. Subramanian et al., (2010) and Zaki et al., (2011) showed that this ratio vary between 0.8 to 7.74 and 2.3 to 5.7, respectively.

Identification of EPS Producing Bacteria

In this work, four isolates were selected for further analysis and biochemical tests (Table 2).

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Table 1. Partial characterization of extracted EPS from twenty bacterial strains.

	Morphology	Gram reaction	Dry weight	EPS concentration	TC (%)	TP (%)	TC/TP ratio	Other components
BS1	rods	Positive	0.15 ± 0.01	4.83 ± 0.02	4.76	1.86	2.56	93.38
BS2	rods	Positive	0.26 ± 0.09	15.79 ± 0.04	3.04	1.11	2.75	95.86
BS3	rods	Negative	0.21 ± 0.09	7 ± 0.03	6.71	2.14	3.13	91.14
BS4	coccus	Negative	0.06 ± 0.03	4.82 ± 0.06	9	2.37	3.8	88.63
BS5	rods	Negative	0.18 ± 0.04	6.11 ± 0.03	6.05	1.64	3.7	92.31
BS6	rods	Positive	0.29 ± 0.01	9.5 ± 0.05	5.89	0.84	7.01	93.26
BS7	rods	Negative	0.29 ± 0.01	9.89 ± 0.04	4.55	0.51	8.9	94.94
BS8	rods	Negative	0.46 ± 0.08	15.33 ± 0.07	3.72	0.26	14.25	96.02
BS9	rods	Negative	0.44 ± 0.04	14.67 ± 0.08	3.89	0.55	7.13	95.57
BS10	coccus	Positive	0.3 ± 0.03	9.78 ± 0.03	6.14	0.41	15	93.45
BS11	rods	Negative	0.06 ± 0.01	2.22 ± 0.02	18.45	3.6	5.13	77.95
BS12	rods	Positive	0.3 ± 0.04	10.67 ± 0.01	6.94	0.84	8.22	92.22
BS13	rods	Negative	0.3 ± 0.05	10.01 ± 0.01	4	1.01	3.97	95.02
BS14	rods	Positive	0.2 ± 0.08	7.01 ± 0.02	5.14	0.57	9.01	94.29
BS15	rods	Positive	0.26 ± 0.07	8.5 ± 0.02	3.88	1.29	3	94.82
BS16	rods	Negative	0.12 ± 0.05	3.89 ± 0.01	7.97	2.31	3.4	89.71
BS17	rods	Negative	0.07 ± 0.04	2.22 ± 0.02	18.9	3.6	5.25	77.5
BS18	rods	Negative	0.24 ± 0.01	7.83 ± 0.01	5.36	1.79	2.99	92.85
BS19	rods	Positive	0.14 ± 0.04	4.67 ± 0.02	7.07	3	2.36	89.93
BS20	rods	Negative	0.34 ± 0.06	11.17 ± 0.01	1.52	1.07	1.42	97.4

Table 2. The biochemical characteristics of EPS producing bacteria isolated from activated sludge at wastewater treatment plant of Bojnourd

	BS2 (*Bacillus muralis*)	BS8 (*Pseudomonas fragi*)	BS9 (*Klebsiella variicola*)	BS20 (*Pseudomonas hunanensis*)
Bacterial morphology	rod	rod	rod	rod
Gram reaction	Positive	Negative	Negative	Negative
Indole production	+	+	+	+
H_2S production	-	-	-	-
MR	-	+	+	+
VP	+	-	-	-
Citrate utilization	-	+	+	+
Sugar fermentation				
Glucose	+	+	+	+
Lactose	+	-	+	+
Mannitol	-	-	-	-
Sucrose	+	-	-	+
DNase	-	+	+	+

These isolates were subjected to 16S rRNA gene identification. The sequencing results obtained showed that these isolates are belonging to two main groups: Gamma-proteobacteria and Firmicutes (Figure 1).

The results of the analysis revealed that BS2, BS8-BS20 and BS9 were characteristic of *Bacillus*, *Pseudomonas* and *Klebsiella*, respectively.

BS2 isolate showed a 98.65% resemblance with *Bacillus muralis* (AJ628748). BS8 showed a 97.92% similarity to *Pseudomonas fragi* (AF094733). The most similarity (99.21%) was found between BS9 and *Klebsiella variicola* (AJ783916).

BS20 showed a 97.67% similarity to *Pseudomonas hunanensis* (JX545210). These bacteria from activated sludge were found capable of producing EPS (Subramanian et al.,, 2010). The EPS producing bacteria have been reported by taxonomically diverse bacteria (Sutherland 2001). The bacteria from the orders and families *Lactobacillales*, *Leuconostocaceae* and *Streptococcaceae* (*Firmicutes*) and *Burkholderiales*, *Pseudomonadales* and *Xanthomonadales* (*Proteobacteria*) are producers of polysaccharides that are employed in a range of different commercial applications (Rehm, 2009; Naessens et al., 2005).

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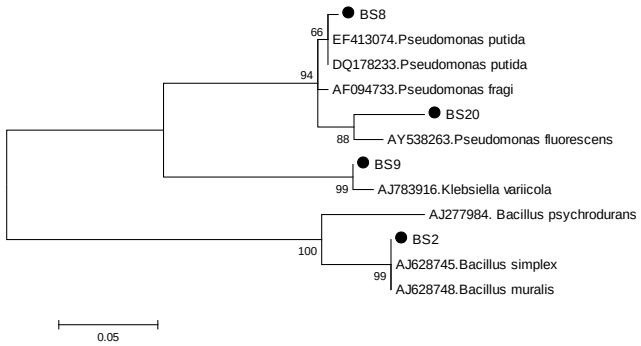

Figure 1: The phylogenetic tree of isolated strains. Clustering was performed using the maximum likelihood method with 1,000 bootstraps.

Effects of Various Parameters on EPS Production

The strains of BS2 (*Bacillus*) and BS20 (*Pseudomonas*) grew in various carbon source. The amount of EPS produced varied while in the presence of different nutrients. The maximum EPS production occured in the presence of glucose as carbon source (Figure 2). These results are similar to the investigation carried out by Mata et al (2006) and Petry et al (2000), in contrast to the results obtained by Pawar et al., 2013. The effect of glucose percentage on the EPS production is shown in Figure 2. The results indicate that the optimum production of EPS for both strains was at 2.5% and 3% glucose. Investigation of various nitrogen sources showed that the maximum EPS production occured in a medium containing yeast extract. A maximum of EPS was also observed when concentration of 0.5% yeast extract was used as nitrogen source. The results suggest that EPS production in the presence of yeast extract increases in comparison with other sources of nitrogen. Wang et al., (2006) have shown that yeast extract was the most effective nitrogen source for the production of bacterial EPS, which might be due to the presence of higher levels of free amino acids, short peptides and growth factors in the yeast extract. The optimum EPS production for two isolates was detected at 30 and 37 °C in mesophilic temperature (Figure 2). The numerous studies have shown that environmental conditions such as pH, temperature and oxygen concentration and nutritional factors such as carbon and nitrogen sources (Cerning, 1990; Looijesteijn, 1999; Vaningelgem et al., 2004) are important factors in the synthesis of extracellular polymeric substances (Conti et al., 1999; Duta et al., 2006).

The results of bioflocculation activity test for four strains is shown in Figure 3. As it can be seen, the most activity for flocculation is belonging to the BS8 and BS9. This behaviour may be related to the chemical composition of these strains, where the TC/TP ratio is higher comparing to two other strains. In fact, carbohydrates play an important role in flocculation of activated sludge, Due to their ability to form bridges between negatively charged groups and divalent cations in the sludge (Higgins and Novak, 1997).

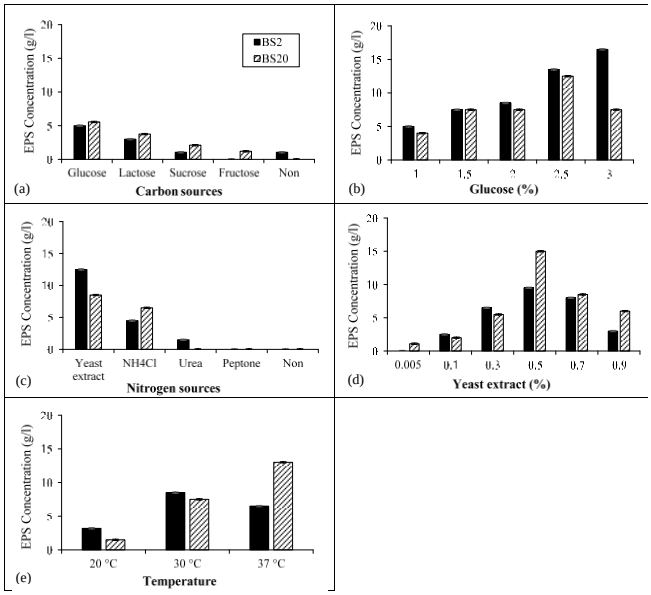

Figure 2. The effect of different variables on EPS production of BS2 (*Bacillus* sp.) and BS20 (*Pseudomonas* sp.).

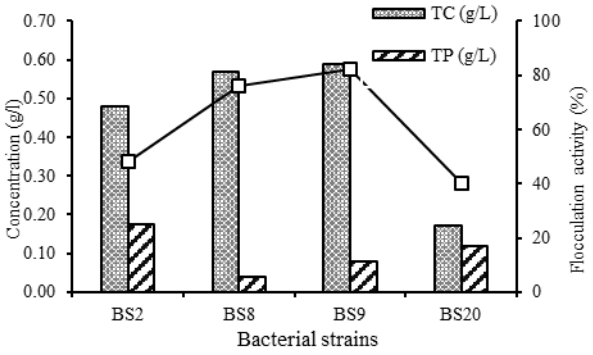

Figure 3. Bioflocculation activity of strains BS2, BS8, BS9 and BS20 in culture medium.

Conclusion

Among 20 species of EPS producing bacteria, four strains were found to produce the highest EPS. Molecular identification showed these bacteria belong to the genus *Bacillus*, *Pseudomonas* and *Klebsiella*. The EPS dry weight and EPS concentration for all bacteria vary between 0.06 and 0.73 g and 72.67-139.34 g/l respectively. EPS concentration in different bacteria and even in a strain are different with respect to the composition of the culture medium and culture conditions such as pH and temperature. Assessment of

environmental conditions showed that EPS production increases at 2.5 to 3% glucose, 0.5% yeast extract and mesophilic temperature. The effect of other environmental factors such as pH, oxygen concentration, incubation time, metal ions, surfactants and NaCl concentration on EPS production have been considered as the effective parameters in EPS production which can be investigated in future studies.

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Research Article

Investigating on the Stability of Peroxidase Extracted from Soybean (*Glycine max* var. Williams) and Effects of Na^+ and K^+ Ions on its Activity

Leila Parsiavash [1], Azra Saboora [1,*], Seyedeh Zahra Moosavi Nejad [2]

[1] *Department of Plant Science, Faculty of Biological Science, Alzahra University, Tehran, Iran*
[2] *Department of Biotechnology, Faculty of Biological Science, Alzahra University, Tehran, Iran*

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Abstract

In the present study, some techniques were used for Soybean peroxidase (SBP) purification including: ammonium sulfate fractionation, DEAE Sephadex anion exchange chromatography and Concanavalin A Sepharose 4B affinity chromatography. Molecular weight of purified SBP was estimated about 44 kDa by SDS-PAGE as a single polypeptide band. The optimal pH and temperature for enzyme activity were found to be 4.5 and 70°C, respectively. The enzyme was more stable in alkaline pH than acidic ones and could tolerate 10 minutes heating in 40-50°C without any loss of its activity. Both NaCl and KCl were found to have significant effects on the enzyme stability, but presence of NaCl was more effective than KCl. Our results showed that after 24 hours incubation of the enzyme in the presence of 20 mM NaCl, more than 60٪ of the enzyme activity was remained while it would fall to 3٪ if incubation was not accompanied by NaCl. Purified peroxidase from seed hull of soybean relative to the other identified peroxidases was more stable, for this reason a lot of benefit will be considered by use of this enzyme in different industry.

***Keywords*:** Enzyme stability, *Glycine max*, Peroxidase, Purification, Soybean

Introduction

Peroxidases are important heme-containing enzymes that have been studied for more than a century. Few enzymes are represented so widely in the scientific and patent literature as peroxidases. These enzymes utilize hydrogen peroxide to oxidize a wide variety of organic and inorganic compounds. Peroxidases are used as reagents for diagnostic test kits, immunoassays and treatment of waste waters (Penel et al., 1992; Vianello et al., 1997). An ideal peroxidase for large-scale biocatalysis as discussed by Liu et al. (1999) would be one that is readily abundant, possesses wide substrate specificity and remains stable over a wide range of pH and temperature. Peroxidase from soybean coat satisfies these criteria. In addition to the vast resource of soybean coats as a major by-product of the food industry, the enzyme is easy to isolate. Soybean seed coat peroxidase (SBP, EC. 1.11.1.7) belongs to class III of the plant peroxidases superfamily and has a wide range of potential applications (Chanwun et al., 2013; Daengkanit and Suvachittanont, 2005). Its structure is a particular interest for engineering purposes and as a model for stable heme peroxidases. There are reports in literature commenting on the purification, activity and application of SBP (Nissum et al., 1998). However, after McEldoon and Dordick (1996) first showed that SBP is substantially more thermo stable than HRP C, considerable interest was shown in this enzyme. The crystal structure of SBP shows that its three-dimensional structure is very similar to HRP C. There are several common features between these two enzymes although SBP has not been studied as thoroughly as HRP C.

In addition to substrates and buffer properties, distinct enzyme tests need a variety of special additives, such as dissociable cofactors or metal ions, which may be directly involved in the catalytic reaction, stabilize the native enzyme structure, or protect it against denaturation. The ions interfere with the weak ionic bonds of proteins. Typical enzymes are active in salt concentrations of 1-500 mM. mono- or divalent cations (M^+ and M^{2+} respectively) neutralize surplus charges at the protein surface and mediate bonds such as salt bridges.

Corresponding authors E-mail:
* saboora@alzahra.ac.ir

On the other hand, heavy metal ions promote oxidative processes, especially with thiol groups, and can affect the native structure. Therefore, addition of the ions is advantageous for some types of enzymes although it may be harmful for others (Bisswanger, 2004). Molecular mechanisms of metal ion coordination and their effects are an important aspect in the characterization of biological macromolecules. Over one-third of known proteins are metalloproteins (Castagnetto et al., 2002). Conceptual associations with protein-metal complexes tend to favor divalent metals of which significance in protein structure and function has been reviewed in detail (Armstrong, 2000) however, several evidence suggest that group I alkali metals (such as Na^+ and K^+) play important role other than nonspecific ionic buffering agents or mediators of solute exchange and transport.

Because of the considerable treatment of peroxidases in different industry and key role of its stability, a lot of interests have been shown by study on this enzyme. In this paper, we explain about purification of a peroxidase enzyme from seed coats of soybean using anion exchange chromatography, affinity chromatography and SDS-polyacrylamide gel electrophoresis technique (SDS-PAGE). Another goal of this research was investigation on stability and activity changes of the isolated fraction during incubation of the purified enzyme under various conditions such as different pH, temperatures, time storage and effect of the presence of Na^+ and K^+ ions in enzyme reaction mixture.

Materials and Methods

Enzyme Extraction and Purification

Soybean seeds (*Glycine max* var. Williams) were supplied from Oil Seed Company (Tehran, Iran) and its coats were obtained by soaking whole seeds in distilled water for approximately one hour. The coats were, then, removed from the seeds, grinded by blender and homogenized in 0.02 M sodium phosphate buffer (SPB, pH=6.8) containing 2% polyvinyl polypyrolidon (PVPP) in a cold room. Homogenate was centrifuged (20,000 *g*, 45 min) and the supernatant was passed through 8 layers of gauze. The 30-80% ammonium sulfate precipitate was prepared based on Hejri and Saboora (2009) and then the pellet dispersed in 0.02 M sodium phosphate buffer (pH 6.8), dialyzed against the same buffer, and applied to a DEAE-Sephadex A-50 ion exchange column (4×17 cm) previously equilibrated with the same buffer. Peroxidase was eluted with a linear salt gradient from 0 to 1.5 M NaCl in 0.02 M sodium phosphate buffer (pH 6.8). Protein fractions which showed both absorbance at 403 nm and peroxidase activity were mixed and pooled as peroxidase extract. This extract was applied to a Concanavalin A-Sepharose 4B column (1.6 cm × 13 cm, Merck) and then equilibrated with a solution containing 0.5 M NaCl, in 0.02 M SPB (pH 6.8). Fraction with peroxidase activity was retained on the column and eluted with a linear gradient from 0 to 1.75 M glucose in 2.5 M NaCl and 0.02 M SPB (pH 6.8). Elutes were pooled, concentrated and then assessed for physical and chemical properties.

Electrophoresis

Purity and molecular weight of the fractions were examined using analytical SDS-polyacrylamide gel electrophoresis techniques (Laemmli, 1970). Protein fractions were run on 7.5% polyacrylamide gel (PAGE) in a cold room (40 mV, 6 h). peroxidase bands were detected on the gel by submerging the gel in a staining solution containing 80 ml of acetate buffer (0.1 M, pH 5), 8 ml of H_2O_2 3% and 4 ml of benzidine 0.04 M in methanol 50% (Van Loon, 1971). Purity and molecular weight of the enzyme was examined on the 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) (Hames and Rickwood, 1990). Proteins were visualized by staining with Coomassie brilliant blue R250. Molecular weight markers were obtained from Fermentas (Lithuania; Mw= 11-170 kDa).

Protein and Peroxidase Assay

Protein concentration was determined according to the method proposed by Bradford (1976) using bovine serum albumin as a standard. Peroxidase activity was determined at 25ºC following the formation of tetraguaiacol (A_{max} = 470 nm, ε = 26.6 $mM^{-1}cm^{-1}$) in a 3 ml reaction mixture containing 950 µl of citrate buffer (0.05 M, pH 4.6), 1 ml of 15 mM guaiacol, 1 ml of H_2O_2 1.6 mM and 50 µl enzyme extract. One unit of peroxidase activity represents the amount of enzyme catalyzing the oxidation of 1µM of guaiacol in one minute (Liu et al., 1999).

Enzyme Stability

The effect of pH on activity and stability of SBP was determined at 25˚C with the use of two buffer systems, 0.05 M phosphate-citrate buffer ranging from pH 2.5 to 8 and 0.05 M glycine-NaOH buffer ranging from pH 8.5 to 9.5.

For determination of enzyme thermostability, 50 µl of enzyme extract was preincubated in citrate buffer

pH 4.5 for 10, 20 and 30 minutes at different temperatures (30–90°C). Then, remaining activity was determined with guaiacol as substrate. The activity was expressed as the percentage of initial enzyme activity (Saboora et al., 2012).
In order to determine the role of monovalent metal ions on the SBP activity, distinct concentrations of NaCl and KCl (2 and 20 mM) were applied within assay mixture. The remained enzyme activity was measured after 1 minute, 2 and 24 hours of incubation.

Statistical Analysis

All experiments were done at least three times. Data were subjected to ANOVA (one way variance analysis) using statistical software SPSS 11 (SPSS Inc, Chicago, USA). Means values were compared by post hoc Tukey test and significant differences determined among the treatments at $p<0.05$.

Results

The result showed that there was 4 mg protein in each gram of soybean coats (*Glycine Max* var. williams). The specific activity of the purified peroxidase was 110.94 U/mg proteins in crude extract which increased to about 1550 U/mg proteins after eradication of impurities and developing of the purification procedure (Table I). The purification method after chromatography on DEAE-Sephadex A-50 and Concanavalin A-Sepharose 4B, respectively, led to 3.8 and 14 fold purification of the peroxidase fraction (Table 1). Finally, 98% of the protein fraction were removed from crud extract and fraction numbed between 30 to70 were collected (Fig. 1, 2).

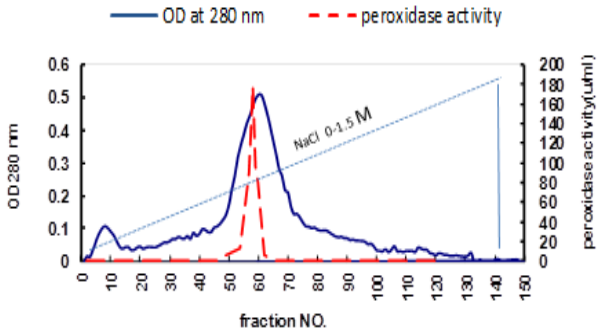

Figure 1. Purification spectra of the soybean seed coat peroxidase. Gradient elution profile from DEAE-sephadex A-50 ion exchange chromaograph column.

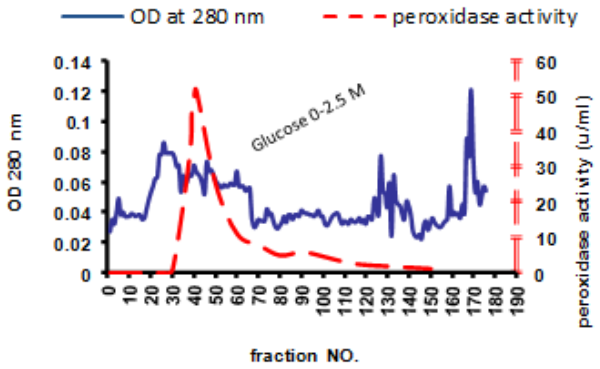

Figure 2. Chromatography of active fractions from spectra of the soybean seed coat peroxidase. Gradient elution profile was obtained from concanavalin A-sepharose 4B affinity chromatography column.

Isolated peroxidase revealed as a single band on SDS-PAGE and had a low molecular weight (44 kDa) according to the migration of the molecular weight markers (Fig. 3). Also, the purity of the peroxidase preparations was determined by measuring the ratio of the heme absorbance absorbance (at 403 nm) to the protein absorbance (at 280 nm). This ratio is denoted the RZ (Reinheistszahl) value. As shown in the Table 1, RZ value increased from 0.3 in crude extract to 0.5 in final preparation of the enzyme.

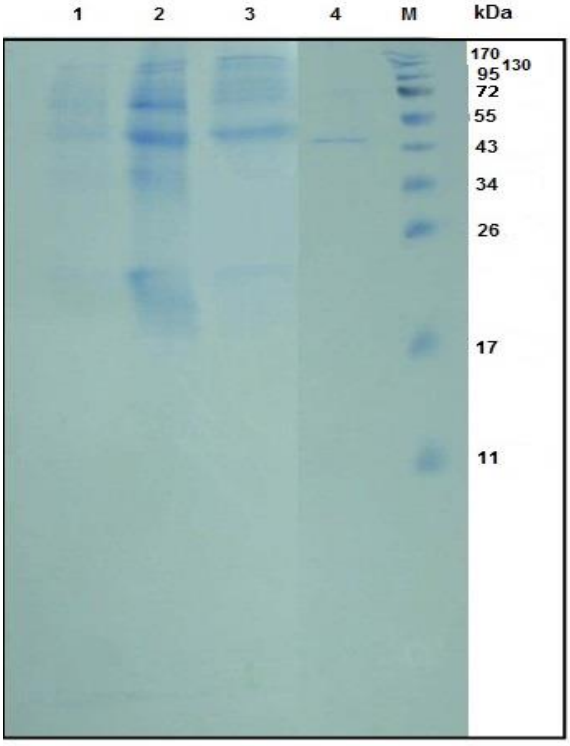

Figure 3. SDS PAGE analysis of soybean peroxidase. Lane M: protein marker, Lane 1: protein extract, lane 2: Amonium sulfate precipitate, lane 3: Elutes from DEAE sephadex chromatography column, lane 4: Elutes from Con A sepharose 4B chromatography column.

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Table 1: Summary of the purification of a peroxidase isoform isolated from seed hull soybean

Purification procedure	Total protein (mg/ml)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)	RZ value
Crude extract	168.48	18690.90	110.94	1	100	0.297
DEAE sephadex column	17.46	7314.23	418.82	3.78	39.13	0.376
Con A sepharose 4B column	0.25	389.05	1549.89	13.97	2.08	0.500

*Units (U) are expressed as µmol of guaiacol oxidized per min and specific activity is expressed as units (U) per mg of protein. Data presented are average of three replicates

The effect of pH on the activity of the purified peroxidase was determined over the range of pH between 2.5 and 9.5. The optimum pH of the purified enzyme was 7. For pH stability, the enzyme activity started to lose at pH below 5.5. Peroxidase activity of the purified fraction was completely abolished after 1h incubation at pH 2.5–3.5 or after 24 h incubation at pH 2.5-5.0 (Fig. 4). Soybean peroxidase was more stable in alkaline pHs than acidic ones.

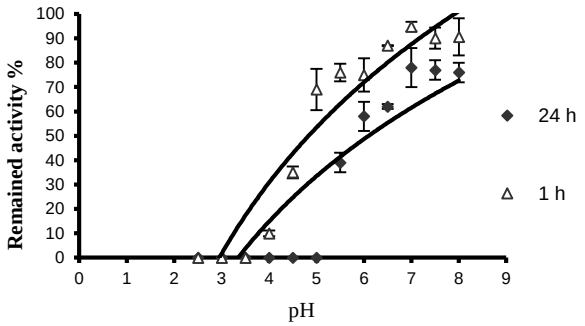

Figure 4. Characterization of the soybean seed coat peroxidase, pH stability profile.

The results of the effect of temperature, 30–80°C, on the enzyme activity demonstrated that the purified enzyme was tolerate for 10 min by warm up at temperatures 40ºC and 50ºC, it exhibited a little inactivation (Fig. 5).

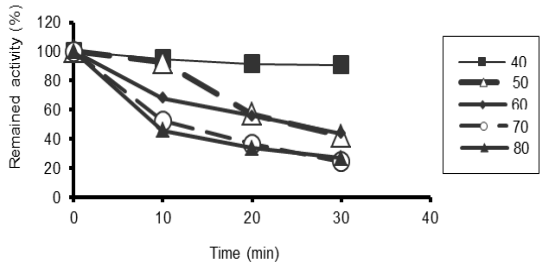

Figure 5. Characterization of soybean seed coat peroxidase, thermostability profile

The purified peroxidase preserved about 50% of the relative activity after 10 min incubation at 70-80°C but the activity was lost more than 67% within 30 minutes (Fig. 5). The enzyme was heat stable at 40°C over a wide range of the experiments and its activity was very low down comparative to 25°C as standard condition. At standard condition, peroxidase activity usually assay under low concentration of the salt (0.05 M sodium citrate or acetate buffer). Compared to the control, our results showed that only one minute incubation of the purified enzyme in the presence of 20 mM concentration of KCl or NaCl led to decrease in peroxidase activity by 4% and 10%, respectively. However, as can be seen in the Table 2, fall in the enzyme activity after 2 hours incubation of the purified enzyme in the presence of sodium chlorid was less noticeable (17.4% for KCl against 11.4% for NaCl). At the same time, peroxidase activity was reduced about 64.6% in the control reaction (Fig 6). Thus, it seems that NaCl is more effective in support of the enzyme stability. Since, the ability of NaCl in keeping the enzyme stable was a bit more than KCl, overnight incubation of Soybean peroxidase was examined in the presence of NaCl. According to the result shown in Fig. 6, it was led to enzyme activity remained up to ٪60 of the control without any impact on pH of assay mixture.

Table 2. Effects of metal ions on the activity and stability of SBP. Both of KCl and NaCl decreased the activity of enzyme in the first minute, but after incubation they had a positive effect in keeping the enzyme stable.

	% Remained Activity	
	after 1 min	**after 2 h**
Control	100 a	35.41 ± 2.46 b
KCl	95.91 ± 4.75 a	82.62 ± 5.14 a
NaCl	89.95 ± 7.38 ab	88.61 a

Data presented are average of three replicates. Different letters denote statistically significant differences by Tukey test at $P < 0.05$

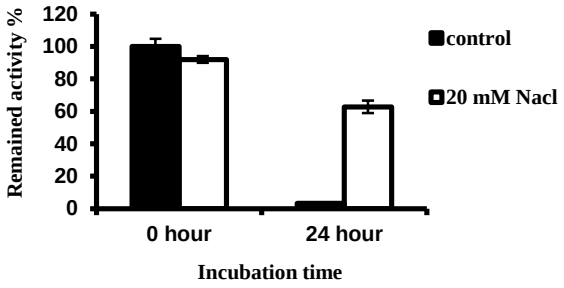

Figure 6. The effect of overnight incubation of the purified SBP in the presence of NaCl.

Discussion

In this study purification was done through three steps, ammonium sulfate precipitation, anion exchange chromatography (DEAE-Sepharose A-50), affinity chromatography (Con A-sepharose 4B) and then followed by SDS-PAGE. The binding of the enzyme to the Con A-sepharose column indicated that the purified peroxidase was a glycoprotein compatible with the previous studies reporting that most of the plant peroxidases were glycoproteins (Joansson et al., 1992; Kvaratskhelia et al., 1997; Passardi et al., 2005). Our purified peroxidase had a molecular weight of 44 kDa as determined by SDS-PAGE while the molecular weights of various peroxidases have been reported to be in the range of 30–150 kDa (Regalado et al., 2004). A similar molecular weight for the purified peroxidase has been previously reported from *Moringa oleifera* leaves and *Ipomoea Batatas* (44 kDa), *Brassica napus* (34-39 kDa) and avocado (40 kDa) (Khatun et al., 2012; Diao et al., 2014; saboora et al., 2012; Singh et al., 2002, Rojas-Reyes et al., 2014). These data are lower than peroxidase from cabbage leaves (67 kDa) and those of *Hevea brasiliensis* cell (70 kDa) (Kharatmol and Pandit, 2012; Chanwun et al., 2013).

Our results revealed that the extracted soybean peroxidase (SBP) enzyme was found to be optimally active at pH 4.5. This is similar to those obtained on POX from *Brassica oleracea capitata L.* (pH 4), *Glycin max* var HH2 (pH 4.6), *Withania somnifera* (AGB 002) and *Hevea brasiliensis* cell (pH 5) (Tabatabaie Yazdi et al., 2002; Liu et al., 1999; Johri et al., 2005, Chanwun et al., 2013). However, these results are lower than those of *Brassica oleracea gongylodes* (pH 8.5) and *Spinacia oleracea* (pH 6.5) (Manzoori et al, Köksal, 2011).

The extracted peroxidase from soybean was optimally active at 70°C. This optimum temperature was lower than those reported for hyperthermostable peroxidase from the *Solanum melongena* (84°C) (Vernwal et al., 2006) and was higher than POX of Turnip root (*Brassica napus* var. okapi) and *Hevea brasiliensis* cell (50 °C) (Saboora et al., 2012; Chanwun et al., 2013).

Also, our results showed that the purified enzyme from seed hull was a thermostable enzyme which had a broad pH-activity profile. Up to pH 8, relative activity retained about 76-78% of the original activity after 24 h incubation. Activity of the enzyme depends strongly on pH of the reaction media for two major reasons: (1) the presence of essential proton-accepting groups in the catalytic center, and (2) maintenance of overall structure of the enzyme. Also, pH dependence of its activity depends on the relative accessibility of reducing substrates and the subsequent electron transfer rate (Veitch, 2004). Kamal and Behere (2003) have reported that the enhanced conformational stability of SBP arises mainly due to the active site architecture in terms of the amino acid interactions with the heme. Therefore, any change around the heme active site brought about by external agents such as pH, solvent, concentration of special ions and a number of factors in reaction mixture can significantly affect on the magnitude of the conformational stability of the enzyme. In most cases, pH-dependent activity changes are reversible, and enzyme incubated in the weakly acid or alkaline range of the titration curve regains its maximum activity when shifted to the optimum pH. However, overall structural changes in the enzyme influenced by pH are often not reversible, and the enzyme does not recover its maximum activity when shifted back to neutral conditions.

The purified peroxidase exhibited high thermal stability in the inactivation profile; its relative activity retained 52-45% of the original activity after heating at 70-80 °C for 10 minutes while at 40 °C, the activity maintained approximately constant during 30 minutes. Most soluble peroxidases, apoplastic and cytosolic isoforms that extracted from several plant tissues have temperature optimas between 30°C and 60°C (Bernards et al., 1999; Loukili et al., 1999).A few number of peroxidases from plants and animals seem to have high temperature optima and show high thermal stabilities (Bakardjieva et al., 1996; Madhavan and Naidu, 2000). Thermal stability of peroxidases has been attributed to the presence of large number of cystein residues in the polypeptide chain (Johri et al., 2005).

Generally, incubation time plays an important role in SBP activity decrease since all treatments were kept in room temperature, but this drop in activity was not statistically significant when the incubation was in the presence of mono-cationic ions (M^+). Regulation of activity through metal ion complexes plays a key role in many enzyme-catalyzed reactions. M^+ lie at a conceptual crossroad between modifier of solvent and metal-cofactor assisting enzyme function. In terms of macromolecular stability, observed differences in valence parameters of M^+ lead to a host of macroscopic features based on hydration properties of these ions (Ahmad et al. 2001). Group IA alkali metals bear a single positive charge with different ionic radius which correspond with significant alteration of ionic volume, hence charge density, and downstream effects upon bonding parameters. Hydration shells of M^+ are dissimilar, and this extends to secondary and tertiary shells of the ion. Na^+ is small enough to bind three or four water molecules with reasonable affinity and result in a larger apparent size in aqueous solution. K^+ favors four or five water molecule coordinated with weaker strength (Page and Cera, 2006). The stability of the alkali ion complexes increased with decreasing ionic radii in aqueous solutions. The stability constant depends on the water activity of any given metal, thereby implicating the differential coordinating abilities of various metal ions at protein surfaces (Sigel et al., 1982).

Conclusion

In conclusion, peroxidase from seed hulls of the soybean grown in Iran can be extracted and purified by means of few classical purification steps. Our study has indicated that there is a single acidic isozyme. Since that last purification steps which were obtained by ion-exchange chromatography and affinity chromatography tend to one peak eluted peroxidase, it can conclude that, enzyme isolation from waste soybean seed coats is relatively easily and cheaply without last stage. Biochemical parameters with respect to enzyme activity and stability revealed its wide possible utilization in biotechnological process involving peroxidases

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Research Article

Supplementary Analysis of Phosphoenolpyruvate Carboxykinase Gene Expression in Developing Seeds of Chickpea

Maria Beihaghi [1], Ahmad Reza Bahrami [2,3], Abdolreza Bagheri [1]*, Mohammad Zare Mehrjerdi [4]

[1] *Department of Biotechnology and Plant Breeding, College of Agriculture, Ferdowsi University of Mashhad, Mashhad, Iran*
[2] *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
[3] *Cell and Molecular Biotechnology Research Group, Institute of Biotechnology Ferdowsi University of Mashhad, Mashhad, Iran*
[4] *College of Agriculture, Shirvan higher education complex, Shirvan, Iran*

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Abstract

A gene of interest in this study is phosphoenolpyruvate carboxykinase (*pepck*), encoding a protein with a substantial role in the gluconeogenesis pathway and in metabolism of nitrogenous compounds in developing seeds of legumes, including amides and ureides which are then transformed into amino acids necessary for the synthesis of storage proteins. Whereas studies on genes contributing to the seed filling in chickpea and its protein content might be valuable in engineering plants with seeds of a higher nutritional value. In order to investigate *pepck* gene expression in different genotypes of chickpea (*Cicer arietinum* L.), four genotypes of chickpea were studied by Real-time PCR and western blot techniques. So results show that *pepck* expresses in high protein genotypes more than low protein genotypes at different growth stages and there was a differential expression of *pepck* gene at different stages of flowering and seed development. The PEPCK was expressed at higher levels during the shoot formation and in developing seeds compared to the flowering and seed formation stages.

***Keywords*:** Chickpea, Protein, Phosphoenolpyruvate Carboxykinase, Real-time PCR, Western blot.

Introduction

Phosphenolpyruvatecarboxykinase (PEPCK), catalyzes the conversion of oxaloacetate to phosphenolpyruvate (PEP) which will be further converted to sugar (Bahrami et al., 2001), and encoding a protein with a important role in the gluconeogenesis pathway in plants. PEPCK is an inducible enzyme, that is only present in certain plant cells, and in many plant and animal tissues and in microorganisms (Walker and Chen, 2002). In plants, PEPCK has been found in the phloem companion roots, cells, stomatal guard cells, the flesh of fruits, simple and glandular trichomes, latex-producing ducts, germinating seeds, developing seeds, and in the leaves of many C4 and CAM plants (Kim and Smith, 1994; Walker and Chen, 2002; Leegood and Walker, 2003). PEPCK is therefore present in many tissues and cell types than was once thought but, in many of these tissues and cells, it is only present under certain conditions, or at certain stages of development (Leegood and Walker, 2003). Also it is well established that PEPCK functions in gluconeogenesis from lipids and proteins in seeds post germination (Leegood and Walker, 1999). Only in some of the plant cell types the function of PEPCK has been clearly established. For some other cells and tissues, there is evidence that PEPCK plays a role in the metabolism of nitrogenous compounds (Chen et al., 2000; Leegood and Walker, 2003; Delgado-Alvarado et al., 2007) .

Metabolism of nitrogenous compounds in legumes developing seeds, including amides such as asparagines which are then transformed into amino acids necessary for the synthesis of seed storage proteins in the grain of some legumes (Aivalakis et al., 2004; Malone et al., 2007; Delgado-Alvarado et al., 2007).

So it is involved in growth, seed filling and amino acid content (especially asparagines metabolism) of pea (*Pisum sativum*) seeds. The seed coat is recognized as a tissue rich in nitrogen transporter

Corresponding authors E-mail:
*abagheri@um.ac.ir

enzymes and invertases which contribute to amino acid and carbohydrate metabolism (Delgado-Alvarado et al., 2007). So the relationship between PEPCK and metabolism of amino acids and amides shows that it is a relatively sensitive enzyme to the presence of nitrogenous compounds in seed coat and cotyledons, with its content being affected by nitrate, ammonium and asparagine in seed coats, but only by asparagine in cotyledons of pea (Delgado-Alvarado et al., 2007).

Chickpea (*Cicer arietinum* L.) is an economically important legume cultivated throughout the world (Chickpea 2005). The aim of this work was to investigate the occurrence of phosphoenolpyruvate carboxykinase (PEPCK) in different stage of reproductive growth of chickpea.

In our previous study (Beihaghi et al., 2009) the protein content percentage was measured in a number of cultivated chickpea genotypes, followed by comparison of the expression levels of PEPCK gene at different stages of seed filling in some of the genotypes by semi quantitative RT-PCR. Also PEPCK mRNA level in different genotypes was determined. two high protein genotypes (MCC053 and MCC458) and Two low protein genotypes (MCC291 and MCC373) out of 25 chickpea genotypes were selected and total RNA was extracted at different seed development stages. Results of semi-quantitative RT-PCR showd that; there was a differential expression of *pepck* gene at different stages of flowering and seed development. The PEPCK was expressed at higher levels during the shoot formation and in developing seeds compared to the flowering and seed formation stages.

In this study, *pepck* expression and the occurrence of PEPCK protein and its activity in different growth stages of chickpea was determined (during both vegetative and reproductive growth) by Real-time PCR and Western blot techniques in different tissues of the plant.

Materials and Methods

conditions of Plant growth

Four chickpea (*Cicer arietinum* L.) genotypes, high protein genotypes MCC458 and MCC053 and low protein genotypes MCC 291 and MCC373 were used in this study. Chickpea seeds were obtained from the Ferdowsi University Genebank (Mashhad, Iran). The germinated seeds were cultured in pots containing equal amounts of clay, leaf compost and sand, and maintained in the glasshouse for 3 months under photoperiod of 14 h light at 25±2ºC and 10 h darkness at 15±2ºC, until the genesis seed stage.

RNA extraction

Total RNA was extracted from different growth stage of such as flowering, sheet formation, seed formation and seed development by RNeasy Plant Mini Kit (Qiagene, ZistBaran, Iran). Fresh tissues were homogenized in liquid nitrogen and other steps were performed according to the manufacturer's instructions.

cDNA Synthesis

Easy™ cDNA Synthesis Kit was used for cDNA synthesis. Reverse transcription was performed in a reaction mixture containing 5 µg of total RNA, 1 µl oligo dT primer and DEPC-treated water Up to 10 µL, then the mixture incubated at 65°C for 5 min and chilled on ice, added 10 µL of RT Premix (2X) and mixed by pipetting gently up and down (total reaction volume 20 µL), incubated 10 min at 25°C and 60 min at 50°C.

Primer design

One specific primer pair (Table1) were designed with Primer5 software using the *pepck* (XM_004509665.2) cDNA sequences for Real-time PCR*;* and *hsp90* (NM_001019786.2). The primers were obtained from Gene Fanavaran (Tehran, Iran).

Table1: Primer sequences used in this study

No	Gene	Primer sequence		Amplicon length, (bp)
1	*pepck*	5' -ATGGTTATCCTCGGCACGC 3'	F	127 bp for Real-time PCR product
		5'-CACCATCTTTGCCCATATTGC 3'	R	
2	Hsp90	5' CAGGAACGACAAATCCGTCA 3'	F	142 bp for Real-time PCR product as control
		5' CTCGTCAATGCTCAGTCCAAG 3'	R	

Real-time PCR

The relative expression levels of *hsp90* and *pepck* genes were determined at various post-inoculation times by Real-time PCR. By establishing a gradient of concentration that covered 150–400 nM for primers and 50–200 ng for cDNA, the dynamic range of cDNA and optimum concentration of primers for Real-time PCR were determined. For all selected genes, the best florescence signal was obtained from 150 ng cDNA. Also, a unique peak was acquired at melting step with 1 µM primers. Each cDNA template was run three times. Gene expression variations were monitored by Rotor Gene 3000 (Corbett research) in 20µl final volume reaction of Real-time PCR containing 3 of cDNAs, 1 µl each primer, and 10 µl 2X SYBR GREEN PCR Master Mix (pars tous).

The Real-time PCR conditions included initial activation step at 95°C for 10 min, denaturation at 95°C for 15s, and combined annealing/extension at 55°C for 30s. Then melting curve analysis of PCR product was carried out at 72°C for 30s foe 35 cycle. Extension step of each cycle and melting step data were collected on the FAM/SYBR channel. The analysis of these raw data was done by *LinReg PCR* program, after exporting them to an Excel worksheet. The PCR efficiency and CT value of each reaction were obtained from Lin Reg PCR software. There after, the relative expression value was calculated using ΔΔCT formula. A quantification of relative expression of *pepck* was obtained by calibrating the expression of this gene with the *hsp90* gene used as a reference gene. The relative expression levels of *pepck* gene in different stages of flowering and seed development of high protein genotype were separately compared with low protein genotype that of control. As shown in Fig. 1, the expression levels in flowering stage of *pepck* gene in MCC291(low protein plants) were set equal to 1 and the cDNA levels of this gene in MCC373, MCC458 and MCC053, in different stage of growth, were calculated relative to this control.

Protein extraction and quantification

Equal amounts (5µg) of each protein sample were electrophoresed on 10% SDS-polyacrylamide gel and electrophoretically transferred onto the PVDF membrane. The transfer buffer contained 12 mM Tris base, 95 mM glycin, and 20% glycerol, and transfer was carried out for 4 h using 90 V constant voltage at 4°C. The membrane was blocked in PBS-Tween (0.1% tween 20) for 20 min and incubated with PEPCK polyclonal primary antibody (kindly donated by prof. R. Leegood, University of Sheffield), diluted 1:5000 in blocking solution 2 h at 30°C. The membrane was washed three times in PBS-Tween (0.1 % tween 20) for 25 min and incubated for 1 h in diluted (1:5000 in the blocking solution). Secondary antibody was horseradish peroxidase- conjugated anti goat IgG (Sigma). Then the membrane was transferred to a shallow tray and 10 µl H_2O_2 (30%) was added to 10 ml of 0.05% DAB (diaminobenzidine) in PBS, and mixed well immediately. DAB was poured onto the membrane and was incubated at room temperature with shaking in dark position(5 min), filter was washed in water, and in PBS. Then the membrane was dried and the photograph of it was taken to provide a permanent record of the experiment.

Results

Real-time PCR

Since *pepck* probably plays an effective role in protein content of plant, the presence of mRNA arising from the *pepck* genes, was determined at different stages of development in two high protein genotypes (MCC053, MCC458) and two low protein genotypes (MCC291,MCC373). The results showed that amount of *pepck* expression was significantly higher at the stage of seed filling than other stages of all genotypes. The lowest levels of expression had been at flowering and seed formation (Fig. 1). Under conditions used in this study, the transition from flowering stage to seed developing stage shows that the activity of PEPCK was increased, PEPCK activity in sheet formation is higher than seed formation in all of the genotypes, but in high protein genotypes these differences were more significant than low protein genotypes (Fig.1). As expected, the expression of this gene was detected in both of two low protein genotypes, but comparatives showed that the measure of *pepck*, was fairly lower in these genotypes. Accumulation of these RNAs in high protein genotypes was significantly higher than that of the control ($P \leq 0.001$) and in low protein genotypes were not so significant (Fig.1). As shown in Fig.1, the expression of *pepck* in growth stages of high protein genotypes; MCC458 was induced at seed development, sheet formation and seed formation (6674 fold , 4473 fold and 3450 fold, respectively) and also MCC053 was induced at seed development 7428 fold, sheet formation 3732 fold and 2413 fold in seed formation stages. So as it shows; it has a few differentiation in expression of this gene between these two genotypes. However, the expression of this gene lower at the stage of flowering (265 fold in MCC458 and 206 fold in MCC053) compared to seed development stages. Also, comparison between high protein and low protein genotypes showed that the high protein genotypes had higher *pepck* expression than low protein genotypes in all flowering and seed development stages (Fig. 1). Thus, in high protein genotypes *pepck* expression was induced, than in low protein genotypes. The result of semi quantitative PCR in the last study (Beihaghi et al., 2009) also showed that The expected band was not amplified in any growth stage of MCC291 and MCC373 genotypes. However by Real-time PCR the differences in expression of *pepck*, visually evident among different growth stages (Fig.1). Also, in order to confirm the amplified bands from RT- PCR, these bands were sequenced and the result of sequencing was blast in NCBI database (Fig.2).

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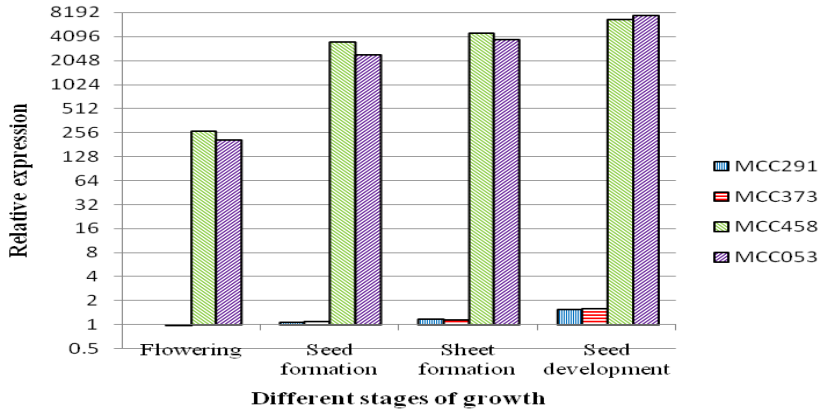

Figure 1. *Pepck* expression at different growth stage of high protein genotypes (MCC053 and MCC458) and low protein genotypes (MCC291 and MCC373). The expression levels of *pepck* gene in flowering stage of MCC291 (low protein genotype) were set equal to 1 as control, and the cDNA levels of this gene in different stage of all of these genotypes have been comparised to this control. Accumulation of these RNAs in high protein genotypes was significantly higher than control ($P \leq 0.001$) and in low protein genotypes were not so significant.

PREDICTED: Cicer arietinum phosphoenolpyruvate carboxykinase [ATP]-like (LOC101503695), mRNA

Sequence ID: refjXM_004512483.1| Length: 2544 Number of Matches: 1

Range 1: 1230 to 1405 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
270 bits(146)	3e-69	166/176(94%)	0/176(0%)	Plus/Plus

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Query  3     CACAAGCATTGATCTTTATCTTGCTAGAAGGGAAATGGTTATCCTCGGCACGCAGTATGC  62
             ||| |||||||||||||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  1230  CACTAGCATTGATCTTAATCTTGCTAGAAGGGAAATGGTTATCCTCGGCACGCAGTATGC  1289

Query  63    CGGGGAAATGAAGAAAGGTCTCTTCAGTGTCATGCAATAGCTCATGCCTAAGCGCCAAAT  122
             ||||||||||||||||||| ||||||||||||| || ||||||||||||||||||||||
Sbjct  1290  CGGGGAAATGAAGAAAGGTCTTTTCAGTGTCATGCATTATCTCATGCCTAAGCGCCAAAT  1349

Query  123   TCTCTCCTTACACTGAGGTTGCATTATGCGGAAAGATGGTGATGTTGCACTCTTCT  178
             ||||||||||||||  ||||||| |||| | ||||||||||||||||||||||||||
Sbjct  1350  TCTCTCCTTACACTCTGGTTGCAATATGGGCAAAGATGGTGATGTTGCACTCTTCT  1405
```

Figure 2. Alignment of amplified bands from Real-time PCR reaction with specific primers.

Western blot analysis

To confidence that if the PEPCK protein express in high protein genotypes more than low protein genotypes; the level of PEPCK protein were just compared in flowering stage of both genotypes. As expected, the expression of this protein was detected in both of them but comparative western blots showed that the level of PEPCK protein was fairly higher in high protein genotype (MCC 053) compared to low protein genotype (MCC 373) (Fig.3).

Figure 3. PEPCK expression at flowering stages of, MCC053 and MCC373 chickpea genotypes, based on western blot experiments.

Discussion

results of this study show that, *pepck* gene is involved in the content of chickpea protein. During development of pea seeds, high amounts of storage protein are rapidly synthesized in the developing cotyledons (Beevers and Poulson, 1972), so these contain the largest seed storage protein in pea. Developing seeds import large amounts of nitrogenous compounds, usually as amino acids, amides or uridines, for the synthesis of seed storage proteins (Murray, 1987; Peoples et al., 1985). In legumes, like other seeds, these high activities of enzymes of nitrogen metabolism found in the seed coat of peas (Sodek et al., 1980). PEPCK was present throughout development, and accumulated in the cotyledons (Delgado-Alvarado et al., 2007). There has been no reported about the role of this gene in protein content of chickpea yet. In plants, phosphenolpyruvatecarboxykinase (PEPCK), encodes a protein with a substantial role in the

gluconeogenesis pathway. It catalyzes the conversion of oxaloacetate to phosphenolpyruvate (PEP) which will be further converted to sugar (Bahrami et al., 2001). Necessary amino acids for protein synthesis in seed, are transported form other organs during the seed development. Glutamine and asparagine are important nitrogen-transport compounds in many plants (Temple et al., 1998). Many amino acids are produced from these amino acids through the transamination and intermediates of the Glycolysis and Citric Acid Cycle (Berg et al., 2002). Glutamine and asparagines, by deamination reaction, produce α-Ketoglutaric acid and oxaloacetate, the two components of the TCA cycle. Accumulation of these components through the large amount of imported amino acids in high protein genotypes, may be needed for PEPCK enzyme to equalize components between TCA cycle and Glycolysis. Also accumulation of storage compounds requires energy and could be limited due to reduced penetration of light particularly and oxygen into the inner parts of the seeds (Galili et al., 2014). In limited oxygen, FAD^+ and NAD^+ components are reduced through the accumulation of $FADH_2$ and $NADH_2$ in TCA cycle and Glycolysis (Berg et al., 2002), and cells may activate the gluconeogenesis pathway to convert accumulating intermediates of these cycle to sugar. Therefore PEPCK is needed for enhancing and equalizing amino acid production in seeds. But maybe the first principle for high protein seeds is larger amounts of amino component that are transported to seeds.

In the present paper by comparison of the expression levels of *pepck* at different seed filling stages in high protein genotypes, it is suggested that PEPCK is involved in increasing of protein content of developing chickpea seeds. Similar results have been reported by Aivalakis and Delgado (2004) on mature seeds of pea and alfalfa has revealed involvement of the *pepck* in grain filling, nitrogen storage, amino acid enrichment, and thus metabolism of storage proteins during seed development. Similarly, our results suggested that in mature seeds of chickpea, the expression of this gene is related to the metabolism of nitrogenous compounds and increasing of seed protein content.` Results of this study suggested that; this gene were regulated differently in two chickpea genotypes (high protein and low protein genotypes). It appears that the significantly higher expression level of *pepck* in genotypes MCC458 and MCC053, further induction of this gene in the growth stage of seed development can effectively enhance rather than other growth stages. Probably, the differential expression of *pepck* is related to its possible role in metabolism of seed components, particularly in determination of the protein content of chickpea seeds. Thus, *pepck* may be has an important role in increase of seed protein content. Further, the results of this study can be useful for constructing transgenic high protein plants in the future.

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Research Article

Warning of Reducing Genetic Diversity of *Abramis brama* (Berg, 1905) in Gilan Coast Using SSR Markers

Zeinab Hosseinnia [1,*], Ali Shabani [1], Hamed Kolangi Miandare [1]

[1]*Department of Fisheries, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran*

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Abstract

Genetic diversity is one of the three levels of biodiversity. The aim of present study was to compare levels of genetic polymorphism between wild Bream populations using seven microsatellite loci. Genetic diversity was investigated by studying samples collected from two regions, the coast of Chamkhale and Bandaranzali of Gilan province. A total of seven microsatellite loci (MFW_7, MFW_{26}, Mcs1EH, Rser10, Bl_1-153, Bl_2-114 and IC_{654}) were used. The average number of alleles in Chamkhale and Bandaranzali coast were 10 and 10.71 alleles, respectively. The numbers of effective alleles were 7.05 and 7.74 alleles in each population. Allele frequency was declined in wild fish due to inbreeding and genetic drift. The mean of observed heterozygosity values were 0.66 and 0.70 in Chamkhale and Bandaranzali coast, respectively. Approximately, all of loci showed deviation from Hardy-Weinberg equilibrium. The genetic similarity and distance between the two populations were 0.316 and 0.684, respectively. The results of Molecular Variance Analysis revealed that genetic diversity within locations was 97 percent, while among them was 3 percent. The F_{st} value was 0.024 that indicates the low genetic differentiation between the two locations which could be explained by the low number of alleles in two populations. Furthermore, the Natural Migration (Nm) between two stations was obtained 16.30. According to the analysis, it seems that *Abramis brama* has not a desirable genetic diversity in the investigated regions.

***Keywords*:** Bream (*Abramis brama orientalis*), Microsatellite, Genetic diversity, Polymorphism

Introduction

There are several species of fishes from Agnatha to Teleostomi in the Caspian Sea, the largest lake in the world .The *Abramis brama orientalis* (Berg, 1906) from order cypriniformes is one of important endemic fish in the southern Caspian Sea. Furthermore, it habitat in the Anzali wetland and the neighboring Caspian coast (Kiabi, Abdoli and Naderi 1999). Considerable differences have been seen among the Caspian coast based on ecological conditions such as temperature, salinity and morphology. These factors can influence the populations of fishes including *A. brama.* Nevertheless, evidence indicate that there is loss genetic diversity among them (Ghasemi et al, 2007; Keivanshokooh and Ghasemi, 2009). Species ability to survive in the nature is determined by genetic variation that affects their ability to adapt environmental changes. Thus, genetic variation is necessary for the species survival and resistance (Bataillon et al., 1996). Microsatellite DNA markers or simple sequence repeats (SSRs) are tandem repeated motif of 1-6 bases found in all prokaryotic and eukaryotic genomes that have been utilized in the assessment of genetic variation and population differentiation studies for a variety of vertebrates (O'Connell and Wright, 1997; Neff and Gross, 2001).This is mainly due to high frequency in genome, Mendelian inheritance, being semi-dominant, small loci size, ease to determine genotype by polymerase chain reaction (PCR) and great polymorphism (Chen et al., 2008; Dewoody and Avise 2000). Recently, microsatellites have been extensively used to evaluate genetic diversity and structure of wild fish species, such as *Liza aurata* (Ghodsi et al., 2011), *Rutilus rutilus caspicus* (Rezae et al., 2009), *Paraschistura bampurensis* (Askari and Shabani., 2013) and *Alosa braschnicowi* (jafari et al., 2014). Since there is no specific SSR primers for these species from gene banks, we used 7 SSR primer pairs from specific species belongs to Cyprinidae. Nowadays, overfishing and degradation of natural environment in Iran resulted in a sharp decline in population of *A.brama.*

Corresponding authors E-mail:
* z.hosseinnia@yahoo.com

Hence, they need to be protected via a restocking program (Coad, 2014).

Restocking programs are a managerial strategy, in which mature individuals are caught from wild and propagated under controlled conditions. Then, fries are released to their natural habitats and this process is repeated next years (Fiumera et al., 1999). Therefore, Iranian Fisheries Organization (Shilat) tried to increase bream populations through the release of artificially bred fry. In 1986, this artificial reproduction performed using only a single pair of spawned for giving eggs and milt. Bream population increased annually using the generations descended from the same pair. For the preventation of loss genetic heterozygosis through inbreeding, Ghasemi et al (2007) and Keivanshokooh and Ghasemi (2009) offer Shilat to import bream stocks from Azerbaijan.

Currently, stock rehabilitation program of *Abramis brama* is conducted by releasing artificially propagated fries with Azerbaijan stocks to the Caspian Sea.

Now, after several years of implementation of conservation program, the aim of this study is to compare the levels of genetic polymorphism of Bream between the coast of Chamkhale and Bandaranzali of Gilan province. The results of this study will have implications for the conservation of genetic resources of this species. Such information is essential for effective culturing, management and conservation of fish populations. Based on this goal and to make decisions on the brood stock to be used for hatcheries, this work could be used as a guideline of the Iranian Fisheries Organization for a successful repopulation.

Materials and Methods

Samples Collection and DNA Extraction

In Autumn 2012, A total of 60 specimens were collected from two station, Chamkhale (37˚ 11′N , 30˚ 10′E) and Bandaranzali (37˚ 28′ N, 49˚ 28′E) costal of Gilan province (30 specimens from each station). Total genomic DNA was extracted from fin pectoral tissue by using the traditional proteinase-K digestion and standard phenol/chloroform techniques (Hillis et al., 1996). Approximately, 100 mg tissue was treated with 25µl proteinase K (10 mg/ml) and 50µl sodium dodecyl sulfate (SDS) (10%) in a 500 µl Sodium Chloride-Tris- Ethylenediamine tetra acetic acid (STE) buffer (0.1 M NaCl, 0.05 M Tris and 0.01 M Na_2EDTA, pH: 8.0) overnight at 37°C. After incubation, DNA was isolated by two phenol-chloroform (25 phenol: 24 chloroform: 1 isoamyl alcohol) steps followed by precipitation with cold absolute ethanol. The quality and concentration of DNA were assessed by 1.0% agarose gel electrophoresis and stored at -20 °C until use (King et al., 2001).

Molecular Analysis

In this study, seven microsatellite markers were amplified by polymerase chain reactions (PCR) using the following primers (Hosseinnia et al., 2014): (Rser10, Ic654, Bl_2-114, Bl_1 -153, Mcs1EH, MFW_7 and MFW_{26}) (Table 1). PCR amplification were carried out in 0.2 ml PCR tubes with an Eppendorf thermal cycler (BIO-RAD, MJ Mini Thermal Cycler). Initial denaturation was achieved at 94°C for 3 min followed by 30 denaturation cycles for 30 s at 94°C, 30 s at the respective annealing temperatures extending to 72°C for 1 min. The final step was extended to 3 min at 72°C. PCR products were separated using 8% polyacrylamide gels stained with silver nitrate (Rajora et al., 2000).

Statistical Analysis

The presence of null alleles was tested using Microchecker version 2.2.3 (Van Oosterhout et al., 2004). Allelic and genotypic frequencies, observed (Ho), expected heterozygosity (He), test for deviations from Hardy-Weinberg Equilibrium (HWE), Fst values and number of migrant (Nm) were calculated by Genealex ver. 6.5 Software (Peakall and Smouse, 2012). Deviation distribution, genetic distance, genetic identity (Nei, 1978), Difference in Ho, He and allelic variation was determined by Wilcoxon's test using statistical software, SPSS v. 16.

Results

In this study, structure and genetic diversity of *A. Brama* was studied at seven microsatellite loci for two stations in Iran. Micro-checker showed no evidence for large allele dropout or stutter-band scoring at any of the five loci but null alleles can exist in all loci. Allele sizes ranged from 100 to 276 bp (Table 1). The Bl_2-114, MFW26 and Rser10 primer showed the maximum allele number (13) compared to other primers. No statistically significant difference was observed between the numbers of alleles per each locus (Table 2). The average number of effective alleles per locus (Ne) in Chamkhale and Bandaranzali were 7.05 and 7.74, respectively, showing no significant difference (P>0.05) between two populations (Wilcoxon-Mann-Whitney test).

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Table 1. Characteristics of *Abramis brama* microsatellite loci used in the present study

Microsatellite locus	Primer sequence (5→3)	N	Size (bp)	Annealing (˚C)	Gene bank ID number
Bl2-114	F:ATCACTGCCATTTTATTA R:CTGCTCCGCTCTGTTCCA	13	168-272	52	FJ468352
Bl1-153	F:GCACAGCTCTAATCGGTCACT R:TATGGTCAAACACGGGTCAA	8	200-276	53	FJ468350
Mcs1EH	F:ACCGGGCTTTAGGCTGTTGGTCA R:TGAGACACATCCCATCACTGCCTACG	9	100-200	59	AY770926
Ic654	F:TGAGCCGACACTAGAAACAGAGC R:GACAAAGTGCAGGCACAGAATG	9	128-172	52	EU252096
MFW7	F:TACTTTGCTCAGGACGGATGC R:ATCCCTGCACATGGCCACTC	7	160-200	61	-
MFW26	F:CCCTGAGATAGAAACCACTG R:CACCATGCTTGGATGCAAAAG	13	100-148	48	-
Rser10	F:TGCGTAATCGTGAAGCGGTG R:GCCACTAAAGCGCAGAAGCC	13	160-232	57	AJ312850

Table 2. Genetic diversity parameters for seven microsatellite loci in *A. Brama*

Location		Bl2-114	BL1-153	MFW7	MFW26	Rser10	IC654	Mcs1EH
Chamkhale	N_a	12	13	8	7	13	8	9
	N_e	9.412	10.127	5.674	4.520	8.333	4.678	6.667
	H_o	0.70	1.00	0.55	0.30	0.65	0.70	0.75
	H_e	0.894	0.901	0.824	0.701	0.880	0.786	0.850
	Fis	0.217	-0.110	0.332	0.615	0.261	0.110	0.118
	P_{Hw}	Ns	Ns	Ns	**	***	Ns	Ns
Bandaranzali	N_a	14	13	10	7	13	9	9
	N_e	10.667	9.639	7.080	5.063	10.127	5.479	6.154
	H_o	0.85	0.85	0.90	0.60	0.85	0.55	0.65
	H_e	0.906	0.896	0.859	0.803	0.901	0.818	0.838
	Fis	0.062	0.052	-0.048	0.252	0.057	0.327	0.224
	P_{Hw}	Ns	Ns	***	***	Ns	***	***

The number of observed allele (Na), Ne, observed heterozygosity (Ho), expected heterozygosity(He) and fixation index (F_{is}) are shown in Table 2. H_o and expected H_e means for all samples were 0.30-1.00 and 0.701-0.906 respectively. In the Chamkhale sample, the mean for heterozygosity and expected heterozygosity values were 0.664 and 0.833, respectively. In the Bandaranzali samples, these values were 0.75 and 0.860, respectively, but there was no significant difference in the average expected and observed heterozygosity between the populations (Wilcoxon-Mann-Whitney test). All seven loci were tested for deviation from the HWE (Table 2). There were significant deviations from Hardy -Weinberg equili-brium at most of the loci in the two populations. After sequential Bonferroni correction (Rice, 1989), among the 14 population-locus tests (2 populations × 7 loci) six out of 14 possible HWE tests were statistically significant. Fixation index (Rst , Fst) were calculated 0.010 and 0.024 between two wild fish populations; respectively. These values were calculated according to the formula [Nm = [(1 / F_{st}) - 1] / 4] between populations. The mean Nm was obtained as 15.509, between Chamkhale and Bandaranzali station, and the minimum and maximum amount calculated for loci MFW_7 and loci Bl_1-153 were 0.138 and 15.978, respectively (Table 3).

Table 3. FST and Nm for tested

	Bl2-114	Bl1-153	MFW7	MFW26	Rser10	Ic654	Mcs1EH
F_{ST}	0.016	0.015	0.038	0.007	0.015	0.016	0.039
Nm	15.319	15.978	6.410	33.289	16.570	14.919	6.081

The average content of F_{is}index was 0.223 across all loci. Furthermore, the analysis of molecular variance and index F_{st} in 99% showed the high genetic diversity (97%) within populations and the low genetic variation among populations (3%) (figure 1).

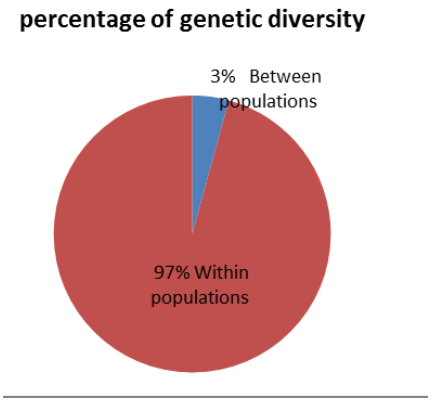

Figure 1. The distribution of genetic diversity on F_{st} standard

Genetic distances and similarities (Nei et al, 1975) computed between the Chamkhale and Bandaranzali fish populations were 0.316 and 0.684, respectively (table 4).

Table 4. Genetic identity (regular font) and genetic distance (bolded font) of *Abramis brama* originated from Chamkhale and Bandaranzali coast

Area	Chamkhale coast	Bandaranzali coast
Chamkhale coast		0.68
Bandaranzali coast	**0.31**	

Discussion

Genetic diversity is important for ecological and evolutionary processes ranging from individual fitness to ecosystem function. Heterozygocity and allele number are important parameters in population genetic variation which determine the ability of organism to compete and survive in natural habitats (Reed, 2009; Hakansson and Jensen, 2005; Frankharn, 2008; Diz and Persa, 2009). In genetic variation studies, allelic richness is more worthy than heterozygocity. In fact, higher allelic richness shows higher effective population size and use of allelic richness is suitable for populations which are treated by selection or conservation programs (Diz and Persa, 2009).

In the case of allele number, Ho and He, the present study is in line with the previous report on *Abramis brama* with the different primers (Keivanshokooh and Ghasemi, 2009). The results of this study indicated that the average number of alleles per locus and observed heterozygosity were comparable, but there was no significanct difference between two populations ($p>0.05$). In our result, observed heterozygosity were higher than the average for freshwater fish (Ho = 0.54 ± 0.25, Dewoody and Avise, 2000). The mean number of alleles are little more than that noted for fresh water fish (Na = 9.1 ± 6.1, Dewoody and Avise, 2000) and anadromous fish (Na = 10.8 ± 7.2, Dewoody and Avise, 2000). The allelic diversity and heterozygosity are both indicative of genetic variation, but allele number is dependent on the effective population size much more than that of heterozygosity (Nei et al., 1975). The Iranian Fisheries Organization produces and releases up to 15 million bream fry into the Anzali wetland annually. Because the hatchery population used for restocking has been founded with a small effective number of parents (Ne), it is likely that these populations have lost some alleles during restocking. Loss of allelic variation has also been reported with (Ghasemi et al, 2007; Keivanshokooh and Ghasemi, 2009). In the genetic population studies allele number is used to determine diversity and is preferred to heterozygosity, because allele number may be lost faster than genetic heterozygosity loss, and low frequency alleles contribute little to overall heterozygosity (Lind et al., 2009). It seems that artificial interbreeding of the Iranian and Azeri broodfish in hatcheries slightly improve the genetic heterozygosity of the Iranian stocks. Unfortunately, number of allels is not satisfying. As showed in table 2, Ho was less than He (Ho= 0.70, He= 0.83). A high gene flow and a low number of specimens are some reasons for a low Ho (Li et al., 2009, Skalla et al., 2004). Eight of 14 tests showed a significant deviation from HWE. Beyond the hypothesis of null alleles, genetic drift and inbreeding are likely to be the causes for deviation from the H–W disequilibrium. In addition, the values obtained from inbreeding index (F_{is}) showed significant defference ($p< 0.05$), indicating increased inbreeding and nonrandom mating (Xu et al., 2001).

Analysis of molecular variance (AMOVA) is a suitable criterion to assess population structure, and to determine the differentiation and genetic similarity between populations (Grassi et al, 2004). According to our obtained F_{st} index, the genetic diversity between the two populations was 3%. The F_{st} index mean was about 0.024, which represents the low differentiation between two populations. According to Wright (1987), F_{st} value of less than

0.05 indicates low differentiation among communities.

In this study, number of migrant's average was reported as 15.50, that was in line with previous study on *Abramis brama* populations which had Nm = 16.30 (Keivanshokooh and Ghasemi, 2009). Li et al. (2007) reported that when Nm> 1, it can be an agent factor for number of migrant and low genetic diversity between populations.

In present study, the genetic identity was 0.68. Shaklee et al, (1982) and Thorpe and Sol-Cave (1994) showed that genetic distance values (Nei, 1972) for cenospecies populations averaged 0.05 (range: 0.002- 0.07), averaged 0.30 for congeneric species (range: 0.03-0.61), and ranged from 0.58 to 1.21 for confamilial genera. The genetic distance between the Chamkhale and Bandaranzali populations falls within the range of congeneric species, suggesting their genetic convergence.

Conclusion

The results of this study suggested that genetic diversity of *A. brama* was rather high in Gilan coasts of the Caspian Sea, but genetic variation between two populations was very low (3%). It seems that mode of reproduction and no appropriate release of larvae are main reasons for genetic differentiation and also, genetic bottleneck in the Bream populations. Restoration of rivers where this species spawned there and providing natural reproduction of brood stocks are best ways for preventing this process.

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Research Article

Biogeography of the Genus *Linaria* (Plantaginaceae) Based on Chromosome Number Data

Massoud Ranjbar * and Samineh Nouri

Department of Biology, Herbarium Division, Bu-Ali-Sina University, Hamedan, Iran

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Abstract

The biogeography of genus *Linaria* was revealed by the available chromosome counts from all over the world. Chromosome numbers of 92 taxa of the genus *Linaria* are included in an on-line karyological database. Furthermore, information about chromosome numbers taken from 374 literature sources was used in this paper. Each database record includes name of taxon, data on chromosome and data on the origin of the material and species-area distribution. Each database also provides a reported chromosome number and place of publication. More than half of the available species in chromosome data are diploid or consist of both diploid and polyploid populations and a few numbers of the species are polyploid. For the first time, in this study, mitosis and meiosis chromosome numbers of 13 populations belonging to three species, namely *L. lineolata Boiss* (2n = 2x = 12), *L. fastigiata* Chav. (2n = 2x = 12) and *L. dalmatica* (L.) Mill. (2n = 2x = 12), were determined by examining anthers. In addition, pollen stainability has been documented in *Linaria* populations in Iran.

***Keywords*:** Chromosome numbers, Karyological database, *Linaria*, Pollen stainability, Polyploidy, Scrophulariaceae

Introduction

Toadflaxes (*Linaria* Miller) is the largest genus of the tribe Antirrhineae (Sutton, 1988). The tribe Antirrhineae as revised by Sutton (1988), consists of 27 genera representing 328 species. *Linaria* posses 200 species world wide, and it is widely distributed throughout the Northern hemisphere with its centre of distribution in the Mediterranean basin and eastern Asia (Hong, 1983). *Linaria* has about 30 species in Iran (Davis, 1982; Sutton, 1988), and comprises annual or perennial herbs with heteromorphic shoots. Flowers are arranged in bracteate racemes and have an accurate personate corolla. *Linaria* species have profuse glandular hairs with unicellular or insert stalks of varying length and heads usually composed of 1-4 cells. Small seeds of *Linaria* are enclosed in capsules, and may or may not be surrounded by an encircling wing. *Linaria* recognized with seven sections (*Diffuse, Linaria, Pelisserianae, Speciosae, Supinae, Macrocentrum* and *Versicolores*), mainly based on seed morphology (In the form and seed), and only useful reliable characters in the separation of these species are the morphology of seed surface and capsule. In this paper, we used cytotaxonomic studies, that can be a powerful tool for investigating different evolutionary trends such as breeding system or polyploidy and hybridization. Cytological studies of *Linaria* species can provide useful information for the assessment of taxonomic relationships (Stace, 2000). The reported chromosome numbers species *Linaria* mainly are 2n = 12, with the exception of one octoploid *L. pelisseriana* (2n = 4x = 48). Valdes and Cabezudo (1977) determined basic numbers x = 6 for most of species of *Linaria* under review. *L. chalepensis* and *L. canadensis* var. *texana* with 2n = 24 (Heitz, 1927, Raven, 1963) and *L. helenica* with 2n = 24, 26 (Contandriopoulos and Yannitsaros, 1975) are tetraploid. The *L. cymbalaria* with seven pairs (East, 1933) and *L. sagittata* with nine pair bivalents in meiosis stage (Dalgaard, 1986) are exceptions in this genus and other species have six pair bivalents in meiosis stage. The small chromosomal variation exists within genus *Linaria* as the members of *L.* sect. *Supinae* subsect. *Supinae* which have been so far cytologically investigated are only diploids with x = 6 and 2n = 12 (Heitz, 1926, 1927a, b; Love and Kjellqvist, 1974; Cardona and Contandriopoulos, 1980; Sutton, 1988; Mayol et al., 1998). It should be mentioned that, the most species have more similar genome sizes. Due to the small chromosomal variation in

Corresponding authors E-mail:
*ranjbar@basu.ac.ir

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Linaria, it is possible to use genome size value to separate some taxa in *Linaria*. An analysis indicates that members of *L.* sect. *Pelisserianae* present the highest value of genome size, as compared to *L.* sect. *Versicolores* present the lowest among other sections of *Linaria* (Castro et al., 2012). The present work aimed to increase the knowledge about cytogenetics and biogeography of the species and comparsion of the basic chromosome numbers and polyploidy levels among the different species, which are distributed throughout the world. Such findings would help us to promote our understanding about the relationships between chromosomal criteria and taxonomic delimitation.

Materials and Methods

Description of Database

There are numerous databases on chromosome numbers available on-line. In this study, on-line database gathers data from published indices to plant chromosome numbers from 1926 onwards (1926-2009). The data are presented as they were published, without any attempt to make a taxonomic revision of the analysed plant material. The database currently contains 92 species taken from 374 literature sources. Also, there are numerous on-line or book reference based databases are available describing the distribution of species. This on-line database or book reference gathers data from published indices on plant distribution from 1827 onwards (1827-2011). Records in the database includes the following data: (1) Name of the taxon (include the name of taxon as published in the original source, the standardized name) (2) Data on the voucher specimen (includes the collector(s) name(s), date of the collection, specimen number and the herbarium collection where it is deposited, species-area distribution, Voucher specimens are deposited in BASU, Hamedan, Iran) and (3) chromosomes (includes mitotic or meiotic chromosome number; ploidy level, detailed data on the locality where material was collected, along with its position in the World Geographical Scheme for Recording Plant Distributions (Brummitt, 2001)). Some chromosome records based online databases and literature are presented in table 1.
[see (http://www.tropics.org/Project/IPCN); (http://www.rjb.csic.es/snapdragons/Paginas/Especie); (http://www. magrama.gob.es/es/biodiversidad/temas/ inventarios); (http://www.botanicalkeys.co.uk/flora/content/species.asp); (http://www.floravascular.com); (http://www.binran.ru); (vargas@rjb.csic.es)]

Table 1. List of *Linaria* species investigated for chromosome number and pollen fertility showing name species,chromosome number, locality, altitude (m), collector, no. voucher and % of pollen sterility.

Species	Voucher number	Locality	Alt. (m)	Collector	Percentage of sterility	2 *n*
L. dalmatica	27675 BASU	Hamedan, Toyserkan, after Shahrestane	1940	RANJBAR and NOURI	11.67	-
L. dalmatica	33895 BASU	Arak to Malayer	1660	RANJBAR and NOURI	3.49	12
L. fastigiata	25021 BASU	Azerbaijan-e gharbi, Bookan toward Miandoab	1870	RANJBAR	3.35	12
L. fastigiata	27029 BASU	Lorestan. Dourud, beginning of Forest Park of Gahar.	1660	RANJBAR	1.73	12
L. fastigiata	27749 BASU	Kermanshah, Sonqor, 10 km after Sonqor	1600	RANJBAR and NOURI	8.45	12
L. fastigiata	29025 BASU	Kordestan, 5 km before of Salavat abad neck	1892	RANJBAR and NOURI	5.87	-
L. fastigiata	27213 BASU	Kermanshah, Sonqor toward qorveh, Kotchkineh village	2062	RANJBAR	18.31	12
L. fastigiata	27632 BASU	Kermanshah, Sonqor, Bavleh village	1840	RANJBAR and NOURI	23.29	12
L. fastigiata	27535 BASU	Hamedan, Babanazar village	1600	RANJBAR and NOURI	33.05	12
L. fastigiata	27188 BASU	Kermanshah, Sonqor toward Qorveh	2050	RANJBAR and NOURI	23.61	12
L. fastigiata	27081 BASU	Kordestan, Salavat Abad neck	1550	RANJBAR and NOURI	-	12
L. fastigiata	30150 BASU	Hamedan to Malayer, Karkan village	1840	RANJBAR	5.24	12
L. fastigiata	26573 BASU	Azerbaijan-e gharbi, Piranshahr toward Oshnaviyyeh	1860	RANJBAR and NOURI	-	12
L. fastigiata	33045 BASU	Hamedan, Alvand Mt, Haydarreh village	1600	RANJBAR	14.35	12
L. lineolata	27473 BASU	Arak, Komeyjan to Vafs, 7 km after Vafs	715	RANJBAR	7.52	-
L. lineolata	25734 BASU	Azerbaijan-e gharbi, Mahabad	1890	RANJBAR	56.63	-
L. lineolata	30626 BASU	Zanjan, Nader Abad village, Deh-jalal neck, 8 km before Soltanieh	1940	RANJBAR	50.30	12
L. lineolata	29133 BASU	Hamedan to Zanjan.Qeydar to Khorkhoreh, 10 km after Qeydar.	1892	RANJBAR and NOURI	16.36	-

Research Article

Cytogenetics

In angiosperms, with the beginning of the first meiotic division, the pollen mother cells are isolated from each other and from the tapetum by the thick layer of callose (Echlin and Godwin, 1968) and they become progressively enveloped in the layer of callose (ß-1, 3-glucan) which characterizes a well-defined stage of pollen grain ontogeny (Waterkeyn, 1962; 1964). The thickness of the callose layer in different species is variable and *Linaria* species have a thick layer of callose in meiotic small cells (figures 1O).

The innermost cell layer of the anther wall in angiosperm species is tapetum layer, (Chaudhury, 1993; Wilson et al., 2001; Kapoor et al., 2002; Sorensen et al., 2002; 2003; Higginson et al., 2003). In *Linaria* species mitotic cells of this layer are different in shape, size and, presence or absence of the vacuole and its number (figures 1Q and R). The tapetum cells are usually bigger and normally have more than one nucleus per cell.

In this study, the mitotic chromosome numbers in anthers of 11 *L. fastigiata* populations, one *L. dalmatica* population and one *L. lineolata* population were studied. Randomly, selected flowers at the appropriate stage were collected for mitotic studies and fixed in 96% ethanol, chloroform and propionic acid (6: 3: 2) for 24 h at room temperature, and then washed and preserved in 70% ethanol at 4 °C until used. Microsporocytes were prepared by squashing and stained with 2% aceto-carmine. Chromosome numbers were counted in five individuals of each population during prophase. The mitotic chromosome association was evaluated in at least twenty cells. Mitotic stages were photographed by a BX-51 Olympus microscope equipped with a 3030 digital camera.

Pollen Fertility

Pollen fertility was studied in 10 populations of *Linaria fastigiata*, two populations of *L. dalmatica* and four populations of *L. lineolata*. Randomly flowers of dry plant were selected and then, pollen fertility results, presented in table 1, obtained using the aceto-carmine method. To determine pollen fertility, darkly stained pollen grains were recorded as fertile and viable, and unstained or very lightly stained ones were considered as sterile or non-viable. Pollen fertility was calculated by dividing the number of viable pollen grains over the total number of grains counted in the scope of view and then, averaging them for all plants in that species.

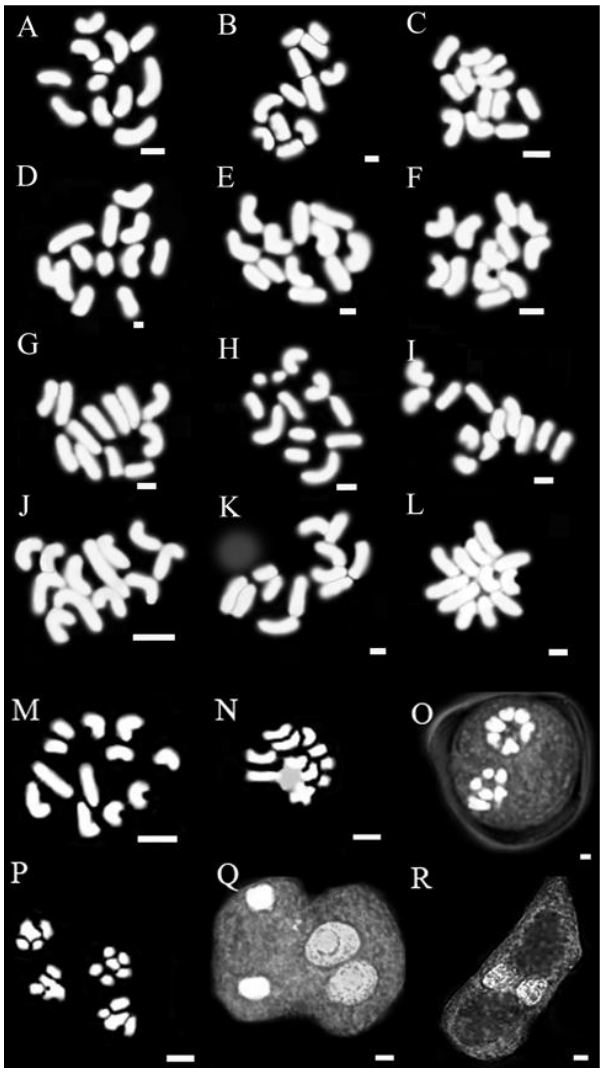

Figure 1. Representative meiotic and mitotic cells in in different species of *Linaria* studied. 2*n* = 2x = 12. (A) Prophase mitotic in *L. fastigiata* 27188, scale bar = 2 µm, (B) Prophase mitotic in *L. fastigiata* 27213, scale bar = 2 µm, (C) Prophase mitotic in *L. fastigiata* 27632, scale bar = 2 µm, (D) Prophase mitotic in *L. fastigiata* 27749, scale bar=20 µm, (E) Prophase mitotic in *L. fastigiata* 27081, scale bar = 1 µm, (F) Prophase mitotic in *L. fastigiata* 27029, scale bar = 50 µm, (G) Prophase mitotic in *L. fastigiata* 27535, scale bar = 2 µm, (H) Prophase mitotic in *L. fastigiata* 26573, scale bar = 50 µm, (I) Prophase mitotic in *L. fastigiata* 25021, scale bar = 20 µm, (J) Prophase mitotic in *L. fastigiata* 33045, scale bar = 2 µm, (K) Prophase mitotic in *L. fastigiata* 30150, scale bar = 1 µm, (L) Prophase mitotic in *L. lineolata* 30626, scale bar = 2 µm, (M) Prophase mitotic in *L. dalmatica* 33895, scale bar = 2 µm, (N) Prophase mitotic in *L. fastigiata* 27188, scale bar = 50 µm, (O) Telophase I meiotic with thick callose layer in *L. fastigiata* 27188, scale bar = 20 µm, (P) Telophase II meiotic in *L. dalmatica* 33895, scale bar = 2 µm, (Q) Telophase I mitotic without vacuoles in *L. fastigiata* 27188, scale bar = 50 µm, (R) Telophase I mitotic with two vacuoles in *L. fastigiata* 27188, scale bar = 50 µm.

Result and discussions

Chromosome Data Analysis

In this paper, the current databases have been described reporting for only 92 species out of 200 total species, counted for the genus *Linaria* throughout the world. Chromosome numbers so far reported for *Linaria* are x = 6, x = 7, x = 9, x = 12, x = 13, but the highest frequent chromosome number reported is 2n = 12 (figures 2, 3B). European taxa are more intensively studied rather than those in other geographical areas. In this study, karyological data for diploidy and polyploidy percentages of the genus *Linaria* are represented as follows: 89% diploid, ca. 11% polyploid: among diploids, 82% (x = 6), 2% (x = 7) and 3% are (x = 9), In *Linaria* species, 3% are polyploid with x = 12 and only 1% probably aneuploid with x = 13 (figure 3B). Base chromosome number 7 was reported only for three species namely *L. cymbalaria* (x = 7), *L. triphylla* (2n = 14) and *L. rubrifolia* (2n = 14). Three species, namely *L. sagittata* (2n = 18), *L. ramosissima* (2n = 18) and *L. lamarckii* (2n = 18) have the base chromosome number 9 and 13 was accounted only for two species, namely *L. odorata* (x = 13) and *L. hellenica* (2n = 26). Sporophytic count was reported 15 only for one species, namely *L. melanogramma* (2n = 15) and the questionable 2n = 6 only for one species, namely *L. nivea* Boiss. and Reut (2n = 6), which is collected from Spain that is cited in IOPB (Goldblatt 1984). Tetraploid level (n = 12) for *L. Simplex* was reported by Ghaffari et al. (2007). According to information, this is the first tetraploid chromosome count for *Linaria* species. However the chromosome number was reported for *L. simplex* only 2n = 12 in ICPN index and in all over the world.

Also, we collected some *Linaria* material from the mountainous regions of Iran and resulted from analysis of mitotic chromosome anthers are summarized in table 1. The meiotic chromosome numbers were studied in 2 populations of *L. dalmatica* pertaining to *L.* sect. *Specioae*, 12 populations of *L. fastigiata* and 4 populations of *L. lineolata* belongs to *L.* sect. *Linaria*. All taxa have a chromosome number 2n = 12 (figure 1). Also in Iranian species, we observed that meiotic cells were smaller and have a thick layer callose and mitotic cells were larger with different forms and sizes (figures 1O, Q and R) surrounded by 1–3 vacuoles. It is evident that after South European, Northern parts of Africa and Middle-East in Asia have more species of *Linaria* respectively. Iberica is a connective bridge between the three continents. It seems that the primary centres of diversity are probably in Iberica with maximum species of the genus. All basic chromosome numbers (x = 6, 7, 9, 12 and 13) of *Linaria* are in southern Europe in the margin of the Mediterranean Sea (Luque and Lifante, 1992). In this article, we cannot separate species of the genus *Linaria* based on chromosome number in the different areas of the world, mainly due to difference between areas in these two cases (figure 2).

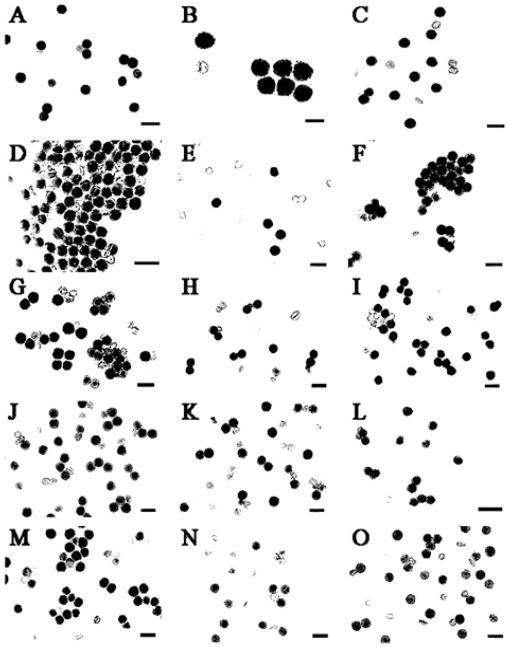

Figure 2. Fertility PMC. Cells in different species of *Linaria*. (A) *L. fastigiata* 25021, scale bar: 10 µm, (B) *L. fastigiata* 33045, scale bar: 20 µm, (C) *L. dalmatica* 33895, scale bar: 200 µm, (D) *L. lineolata* 25734, scale bar: 10 µm, (E) *L. fastigiata* 27535, scale bar: 20 µm, (F) *L. fastigiata* 27029, scale bar: 200 µm, (G) *L. lineolata* 30626, scale bar: 20 µm, (H) *L. lineolata* 29133, scale bar: 200 µm, (I) *L. fastigiata* 27632, scale bar: 20 µm, (J) *L. fastigiata* 27213, scale bar: 20 µm, (K) *L. fastigiata* 27188, scale bar: 200 µm, (L) *L. lineolata* 27473, scale bar: 10 µm, (M) *L. dalmatica* 27675, scale bar: 20 µm, (N) *L. fastigiata* 29025, scale bar: 20 µm, (O) *L. fastigiata* 27749, scale bar: 20 µm.

There are n = 6 in all ten geographical zones ((Europe-Asia: South-Center Europe, Northern parts of Europe-Siberia, South West Asia, Far East), (America: Northern parts of America, Canada, southern parts of America), (Africa: South-center, North) and Australia), n = 7 in the Northern hemisphere New World and the Mediterranean margin, n = 9 in the Mediterranean margin and Southwest Asia of Old World and ploidy levels of n = 12, 13 in the Mediterranean margin, Southwest Asia and the Northern hemisphere New World. We have proposed that speciation in *Linaria* cannot be very old and with the exception of n = 6, other chromosome numbers already have been

engendered and until now an opportunity for distribution of the genus has not been found. However, there are 82% of diploid species with x = 6 and less than 20% with other levels (x = 3, 7, 9, 12, 13, 15) in this genus (figure 3).

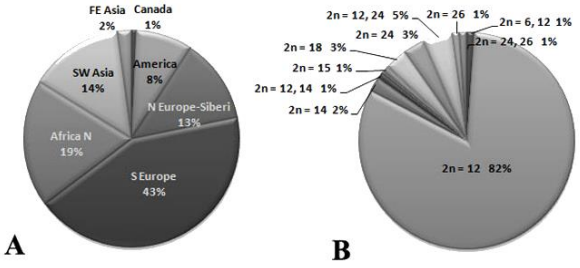

Figure 3. (A). Percent frequency variable chromosome numbers in all over world, (B). Percent distribution species within the genus *Linaria* from all over the world for the species have chromosome report.

Pollen Fertility

For the plant taxonomists pollen fertility is valuable in attempting to distinguish putative hybrids from the parent plants and is also useful to determine the degree of fertility in those plants that are grown under unfavourable conditions (Lawrence, 1969). Several investigators have presented evidence indicating a correlation between the percentage of normal pollen grains and the ultimate fertility of hybrid plants (Poole, 1931). However, pollen fertility can generally high in both diploids and tetraploids and cannot distinguish diploid from polyploid (Qureshi et al., 2009).The assumption of these estimates is that pollen grains that are potentially fertile (its mean that they have no problem of sterility because of meiotic abnormality) will absorb the stain and the sterile ones will appear hollow in the microscope (figure 4). Pollen stainability has measured since 1.73 up to 56.63 in 16 diploid populations include 10 populations of *L. fastigiata* that pollen stainability has measurements consist of: (1.73, 3.35, 5.24, 5.87, 8.45, 14.35, 18.31, 23.29, 23.61, 33.05) with average 13.72% of this species. Two populations of *L. dalmatica*, which pollen stainability has measurements consist of: (3.49, 11.67) with average 7.58% of this species and in four populations of *L. lineolata* pollen stainability has measurements consist of: (7.52, 16.36, 50.30, 56.63) with average 32.70% of this species, all investigated species were diploid (table 1). In spite of our interest to do a review for polyploids population, we couldn't find any polyploid population in Iran.

Note: We have prepared two analyses, one based on the distribution of all *Linaria* species in the world (Fig 4), and another based on the distribution of species that have reported chromosome numbers which are presented in the literature (Fig 3).

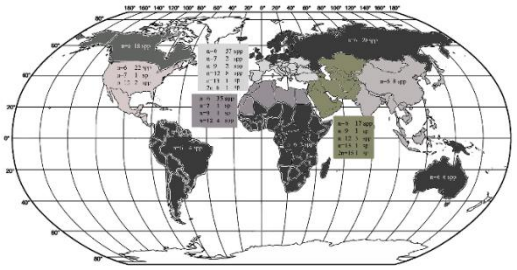

Figure 2. Map ploidy distribution within the genus *Linaria* in all over the world (*n* = 6, *n* = 7, *n* = 9, *n* = 12, *n* = 13, 2*n* = 6, 2*n* = 15), for all regions that name species have reported.

Distribution of the Genus *Linaria*

Interpretations of the cytogenetic differences between Old and New World of *Linaria* have not varied widely. In this paper, distribution of genus *Linaria* studied in ten zones (Europe-Asia: South-Center Europe, Northern parts of Europe-Siberia, South West Asia, Far East), (America: Northern parts of America, Canada, southern parts of America), (Africa: South-center, North) and Australia. Europe-Asia has 130 species of the genus *Linaria*: (28 species in Northern parts of Europe-Siberia, 8 species in the Far East, 23 species in South West Asia and 71 species in South-Center Europe). It is also continental America has 47 species: (18 species in Canada, 4 species in southern parts of America, 25 species in Northern parts of America) and continental Africa has 43 species: (2 species in South Africa and 41 species in Northern parts of Africa). The latter continental Australia has 6 species of this genus (figure 2). Also Mediterranean region has presently 135 species (South-Center Europe (71 spp.), Northern parts of Africa (41 spp.) and South West Asia (23 spp.). Distributions of *Linaria* species that have chromosome reports studied in 7 zones: South-Center Europe, Northern parts of Europe-Siberia, South West Asia, Far East, Northern parts of America, Canada and Northern parts of Africa. Among taxa that have chromosome number records, 56% in Europe-Siberia (Northern parts of Europe-Siberia (13%) and South-Center Europe (43%)); 16% in Asia (Far East (2%) and South West Asia (14%)), 9% in continental America (America (8%) and Canada (1%)) and 19% of species occur in Northern parts of Africa (segmentations of geographic areas in figure 3A are according to author). It should also be mentioned that Mediterranean region (South-Center Europe (43%), Northern parts of Africa (19%) and South

West Asia (14%)) have 76% of the chromosome number records.

Biogeography

The geographical distribution of the taxa belonging to the genus *Linaria* was obtained from online databases and atlases mainly based on their localities taken from floras and literatures that mapped here (Hamdi and Assadi, 2011; Boissier, 1888; Borgmann, 1964; Davis, 1982; Dumortier, 1827-1830; Hansen and Sunding, 1993; Hooker, 1884; Kuprianova, 1950; Parsa, 1949; Press and Short, 1994; Saez Bernal, 2009; Sanchez-Gullon et al., 2006; Valdes, 1978-1986; Vicioso, 1946) (figure 4). Cytological evidences have not indicated a distinction between Old and New World of *Linaria*. Based on a recent survey (table 2, figure 3B) of the 92 old species for which chromosome numbers have been determined, counts of x = 6 have been reported for 83 species, n = 7 for 3 species, n = 9 for 3 species, n = 12 for 9 species and n = 13 for 2 species that some of these are questionable due to discrepancies in samples from the same species and among authors. Among 7 geographic zones for the genus *Linaria* that have reported chromosome numbers in the literature, 71 species occur in southern parts of Europe which 80.28% of species with n = 6, 2.81% of species with n = 7, 2.81% of species with n = 9, 11.26% of species with n = 12 and 1.40% of species with n = 13. 41 species of the genus *Linaria* occur in Africa, including 85.36% of species with n = 6, 2.43% of species with n = 7, 2.43% of species with n = 9 and 9.75% of species with n = 12. Among the 23 bechanced species of *Linaria* in Asia, 73.91% of species with n = 6, 4.34% of species with n = 9, 13.04% of species with n = 12, 4.34% of species with n = 13 and 4.34% of species with 2n = 15. New world species having n = 6, 7 and 9 and higher chromosome numbers are derived from species with n = 12. 25 species of *Linaria* occur in America, including 88% of species with n = 6, 4% of species with n = 7 and 8% of species with n = 12. 28 species of the genus *Linaria* arise in the centre and Northern parts of Europe and Siberia, that all of the species have n = 6. 8 species of *Linaria* bechance in the Far East, which n = 6 have been reported for all of them. 18 species of *Linaria* arise in Canada and n = 6 have been reported for all of them. Among three geographic zones for the genus *Linaria* with no reported chromosome numbers, two species occur in southern parts of Africa (with n = 6 in zones which have chromosome number report), four species of the genus *Linaria* occur in southern parts of America (with n = 6 for all of the species in zones which have chromosome number reports), and 6 species of the genus *Linaria* occur in Australia (with n = 6 for all of the species in zones which have chromosome number report).

Chromosome Evolution in *Linaria*:

Linaria distributed primarily in regions of the Northern hemisphere. The Mediterranean basin only encompasses 1.6% of the earth's surface (Melendo et al., 2003). A remarkable spatial and temporal complexity made the Mediterranean basin an ideal geographic framework for this approach. The complex Iberian orography may have allowed partial differentiation of lineages in allopatry that may have been the framework for *Linaria* and many other southern European plant groups (Feliner, 2011). The *Linaria* distribution centre is placed on the Iberian Peninsula of South Western Europe and it is probably evolved from the Mediterranean in response to various selection pressures toward different regions of the World (Valdes, 1970). There is no regular pattern in the distribution of the genus, as in this research, we cannot separate species of *Linaria* based on either distribution section and chromosome number in the world, because there is not difference between areas in these two cases. The commonly reported base chromosome in the genus are 6, 7 and 9, with a polyploid series of tetraploid being developed on x = 6. There are a few previous reports of tetraploid numbers based on x = 6. The chromosome number n = 12 was reported for eight species, namely *L. kocianovichii*, *L. miller*, *L. hellenica*, *L. chalepensis*, *L. pelisseriana*, *L. canadensis* var. *texana* (L.), *L. angustissima* and *L. arcusangeli* that most of the species are annual and only one species is perennial, since annual species have higher selfing rates than perennials. Generally, polyploidy is one of the prominent and significant forces in plant evolution (Leitch and Bennett, 1997; Otto and Whitton, 2000; Soltis and Soltis, 2000; Wendel, 2000; Liu and Wendel, 2003). It is a process of genesis and maintenance of plant diversity play a role in Scrophulariaceae *s.l.* in the Iberian Peninsula, but apparently is not among the main mechanisms of current speciation in now *Linaria* places in Plantaginaceae. (Castro, 2011). However, sociological studies of the *Linaria* species growing in Iran, also indicate that polyploidy are not a common phenomenon within this genus (Ghaffari 2006, Ghaffari et al. 2007). In studies conducted so far in *Linaria*, all polyploid species are in the Mediterranean margin and only one species is in the New World (table 2).

Table 2. Chromosome number reports in the genus *Linaria* (* = diploid, ** = aneuploid, *** = polyploid, 4* = haploid, 5* = hybrid)

Species	Section	A reference article reports Chromosome number	Locality species	Locality species that chromosome number reported	2N
Linaria acutiloba Fisch. ex Rchb.	?	http://www.binran.ru	Asia	Asia: Russia, East Siberia	12*+ 0-4B
L. aeruginea (Gouan) Cav.	*Supinae*	Chater et al. 1972, Cardona and Contandriopoulos 1980, Love and Kjellqvist 1974	N Europe	SW Europe	12*
L. albifrons (Sibth. and Sm.) Spreng	*Diffusae*	Chater et al. 1972, Snogerup 1985, Diaz Lifante et al. 1992	Asia, Europe, Africa, Australia	SW Europe	12*
L. algarviana Chav.	*Versicolores*	http://www.rjb.csic.es/snapdragons; http://www.floravascular.com, Chater et al. 1972, Viano 1974, Viano 1973	Europe	SW Europe	12*
L. alpina (L.) Mill.	?	http://www.rjb.csic.es/snapdragons; Chater et al. 1972, Murin and Paclova 1986, Dobea et al. 1996, Heitz 1927a, Favarger and Huynh 1964, Lovka et al. 1971, Fernandes et al. 1977	Asia, Europe	C and S Europe	12*
L. amethystea (Lam.) Hoffmanns. and Link	*Supinae*	Chater et al. 1972, Pastor et al. 1988, Fernandes et al. 1977, Valdes 1969, Van Loon and De Long 1978	Europe, Africa	SW Europe, Africa	12*
L. amoi campo ex Campo and Amo	*Supinae*	http://www.rjb.csic.es/snapdragons, Chater et al. 1972, Valdes 1969, 1970b	Europe, Africa	SW Europe	12*
L. angustissima (Loisel.) Borbás	*Linaria*	http://www.rjb.csic.es/snapdragons (2*n* = 12), http://www.floravascular.com (2*n* = 12), Chater et al. 1972 (2*n* = 12), Uhrikova et al. 1983 (2*n* = 12), Majovsky 1974 (2*n* = 24)	Europe, America, Asia	Mediterranean region, SW Asia	12*, 24***
L. anticaria Boiss. and Reut.	?	http://www.rjb.csic.es/snapdragons; Luque and Lifante 1991, Heitz 1926; 1927a, Valdes 1970b	Europe	SW Europe	12*
L. arcusangeli Atzei and Comarda	?	Bacchetta 2001 (2n = 12), Atzei and Camarda 1984 (2*n* = 24)	Europe	Europe: Mediterranean	12*, 24***
L. arenaria DC.	?	http://www.magrama.gob.es.gob.es/es, Serrano Perez and Carvajal Villaverde 2004	Europe	SW Europe	12*
L. arvensis (L.) Desf.	*Supinae*	http://www.rjb.csic.es/snapdragons, Chater et al. 1972, Heitz 1927a, Löve and Kjellqvist 1974, Van Loon and Dejong 1978	Europe, Africa, Asia	S, W and C Europe, NW Africa, SW Asia	12*
L. balbata Dieter	?	Matsura et al. 1935	Asia: Japan	Asia: Japan	12*
L. bipartita (Vent.) Willd.	*Macrocantrum* or *Versicolores*	Verma and Dhillon 1967, Heitz 1926, Chandran and Bhavanandan 1983, Nazeer et al. 1980	Asia, Africa	N African: W Morocco	12* + 0-1B
L. broussonnetii Chav.	*Versicolores* ?	East 1933	Àfrica, America	America: Massachusetts	12*
L. bubanii Font Quer	*Supinae*	Chater et al. 1972, Montserrat Marti 1981-1982	Europe	SW Europe	12*
L. caesia DC. ex Chav.	*Supinae*	http://www.rjb.csic.es/snapdragons, Valdes 1970b, 1973, Chater et al. 1972, Van Loon and De Long 1978	Europe	SW Europe	12*
L. canadensis (L.) Dum. Cours.	*Macrocantrum* or *Versicolores*	Kapoor et al. 1987 (2*n* = 12) (report for *Nuttallanthus canadensis* (L.) D. A. Sutton, Raven 1963 (2*n* = 24)	America: Mexico and Texas, Havai Asia cult.	America	12*, 24***
L. capraria Moris and De Not.	?	http://www.rjb.csic.es/snapdragons, Heitz 1927a, 1927b, Signorini et al. 2001	Europe	S Europe	12*
L. cavanillesi Chav	*Diffusae*	Boscaiu et al. 1997	Europe	SW Europe	12*
L. chalepensis (L.) Mill.	*Macrocentrum*	http://www.floravascular.com, http://www.rjb.csic.es/snapdragons, Heitz 1926, 1927b, Ghaffari 2006, Chater et al. 1972	Europe, Asia, Africa, Australia	Mediterranean region	24***
L. clementei Haenseler ex Boiss.	*Versicolores*	http://www.floravascular.com, Chater et al. 1972, Viano 1979	Europe	SW Europe	12*
L. corifolia Desf.	?	http://www.rjb.csic.es/snapdragons, Viano 1972	Asia	SW Asia, Russia	12*
L. cymbalaria (L.) Mill.	?	East 1933, Chatterjee et al. 1989	America: Mexic Europe	America: Massachusetts	14**

L. dalmatica (L.) Mill.	*Linaria*	Vujnovic and Wein 1996, Gervais 1981, Heitz 1926, Valdes 1970b, Chuang and Heckard 1992	America, SW Asia, S, C Europe, far East, Australia	America: Canada, SE, SC Europe, SW Asia	12*
L. debilis Kuprian	*Linaria*	Rostovtseva et al. 1981	Asia	Asia: Siberia	12*
L. diffusa Hoffmanns. and Link	*Supinae*	http://www.rjb.csic.es/snapdragons, Fernandes et al. 1977, Chater et al. 1972	Europe	N, C and SW Europe	12*
L. elegans Cav	*Versicolores*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Heitz 1926, Chater et al. 1972	N Europe, Asia, Africa	Mediterran ean region, SW Asia	12*
L. faucicola Leresche and Levier	?	Valdes 1978	Europe	SW Europe	12*
L. flava (Poiret) Desf.	?	Verlaque et al. 1992	Europe, Africa	Europe: Zurich	12*
L. genistifolia (L.) Mill.	*Speciosae*	http://www.rjb.csic.es/snapdragons, Buttler 1981, Jasiewicz and Mizianty 1975, Kiehn et al. 1991, Van Loon and Setten 1982, Dobea et al. 1997, Javourkova-Jarolimova 1992, Heitz 1926, Valdes 1970b, Van Loon and Van Setten 1982	Europe, Asia: Far East, America	SE Europe, Asia: Anatolia, Russia	12*
L. glacialis Boiss.	*Supinae*	Küpfer 1968	Europe	SW Europe	12*
L. glauca (L.) Chaz.	*Supinae*	Baltisberger and Charpin 1989, Valdes 1978	Europe	SW Europe	12*
L. haelava (Forssk.) Delile	?	Diaz Lifante et al. 1992	Africa, SW Asia, Australia	Mediterran ean	12*
L. hellenica Turrill	?	http://www.rjb.csic.es/snapdragons (2*n* = 24, 26), Contandriopoulos and Yannitsaros 1975 (2*n* = 24, 26)	Europe	SW Europe	24**, 26***
L. heterophylla Desf.	?	East 1933, Rossitto et al. 1983, Devesa et al. 1984, Silvestre 1991, Galland 1988	Europe, Africa, Asia, America: Massachusetts	America: Massachusetts	12*
L. hirta (L.) Moench.	*Diffusae*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Viano 1973; 1974, Löve and Kjellqvist 1974, Chater et al. 1972, Luque and Lifante 1991	Europe, Africa, Asia	C, S Europa and Mediterranean, NW Africa, SW Asia	12*
L. huteri Lange	*Supinae*	Chater et al. 1972, Viano 1973	Europe	Europe and Mediterranean	12*
L. incarnata Spreng.	*Versicolores*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Chater et al. 1972, Silvestre 1991, Valdes 1969; 1970b, Fernandes et al. 1977	Europe, N Africa, America	SW Europe and Mediterranean	12*
L. japonica Miq.	*Diffusae*	Karol Marhold 2008, Probatova and Sokolovskaya 1981	Asia: Russia, Far East	Asia: Russia, Far East	12*
L. kocianovichii Asch.	?	Murin et al. 1999	Europe	S, C Europe	24***
L. lamarckii Rouy	?	Caixinhas et al. 1991	Europe	SW Europe	18**
L. Lineolata Boiss.	*Linaria*	http://www.rjb.csic.es/snapdragons, Heitz 1927a	Asia	SW Asia	12*
L. macroura (Bieb.) Link	*Linaria*	http://www.rjb.csic.es/snapdragons, East 1933, Heitz 1926	Asia, Europe America: Massachusetts	America: Massachusetts, SE Europe, Asia: Russia	12*
L. maroccana Hooker fil.	*Versicolores*	http://www.rjb.csic.es/snapdragons, East 1933, Heitz 1926	N Africa, Europe, Americaee: Massachusetts, Australia	America: Massachusetts, N Africa:	12*
L. melampyroides Kuprian.	*Linaria*	Probatova 2006	Asia: Siberia	Asia: Moscow, Leningrad	12*
L. melanogramma Rech. f. and al.	?	Aryavand 1983	Asia	Asia: Iran	15** or 5*
L. meyeri Kuprian.	*Linaria*	Gagnidze 2006	Asia	Asia: Moscow, Leningrad	12*
L. michauxii Chav.	*Linaria*	http://www.rjb.csic.es/snapdragons, Aryavand 1977	Asia	Asia: Iran.	12*
L. micrantha (Cav.) Hoffmanns. and Link	*Supinae*	http://www.rjb.csic.es/snapdragons, Löve and Kjellqvist 1974, Fernandes et al. 1977, Chater et al. 1972, Valdes 1978	N Europe, Africa, Asia	S Europe, Mediterranean, SW Asia, N Africa	12*
L. miller Gard	?	http://www.rjb.csic.es/snapdragons	Europe, Asia, Africa	Europe, Asia, Africa	12*, 24***

L. nivea Boiss. and Reut.	*Speciosae*	http://www.floravascular.com (2*n* = 12), Chater et al. 1972 (2*n* = 12), Elena Rossello et al. 1984, 1986 (2*n* = 12), Viano 1975 (2*n* = 6)	N Europe, Africa, Asia	Mediterranean region and Europe, Africa, SW Asia	12* 6?[4*]
L. oblongifolia Boiss. and Reuter	*Supinae*	Chater et al. 1972, Viano 1979, Valdes 1969	N Europe	SW Europe	12*
L. odorata M. Bieb.	*Linaria*	Chatterjee et al. 1989	Europe, Asia	Asia: Himalayan	26***
L. oligantha Lange	*Supinae*	Chater et al. 1972, Valdes 1978	Europe	SW Europe	12*
L. orbensis Carretero and Boira	*Supinae*	Chater et al. 1972, Mayol and Rosello 1998	Europe	SW Europe	12*
L .pallidiflora (Lam.) Valdés	*Linaria?*	http://www.floravascular.com	Europe: Slovenská	SW Europe	12*
L. pancicii Janka ex Nyman	?	East 1933	Asia: Alaska, America: Massachusetts	Americaee: Massachusetts	12*
L. pedunculata (L.) F. Dietr.	*Versicolores*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Chater et al. 1972, Vogt and Oberprieler 1994, Viano 1975; 1979	Europe, N Africa	Mediterranean region Europe, NW Africa	12*
L. pelisseriana (L.) Miller	*Pelisseriana e Supinae*	http://www.rjb.csic.es/snapdragons, http://www.botanicalkeys.co.uk/flora, http://www.floravascular.com, Ward et al. 2009, Chater et al. 1972, Strid and Franzen 1981, Verlaque et al. 1997, Larsen and Laegaard 1971, Dahlgren et al. 1971, Strid 1981	Europe, Africa, Asia, Australia, America	C and E Mediterranean region, W Europe, N. Africa	24***
L. peloponnesiaca Boiss. and Heldr.	?	Baltisberger 1987, Baltisberger and Baltisberger 1995, Strid and Franzen 1981	Europe	S. Europe	12*
L. peltieri Batt.,	?	http://www.rjb.csic.es/snapdragons, Reese 1957	Europe, Africa	SW Europe, N. Africa	12*
L. perezii J.Gay	?	East 1933	Europe, America: Massachusetts	Americaee: Massachusetts	12*
L. platycalyx Boiss.	*Supinae*	Chater et al. 1972, Aparicio Martinez 1993	Europe, Africa	SW Europa	12*
L. polygalifolia Hoffmanns. and Link	*Supinae*	http://www.rjb.csic.es/snapdragons, Chater et al. 1972, Castroviejo and Lago 1990, Valdés 1973, Viano 1974, Fernandes and Queiros 1971, Fernandes et al. 1977, Lago Canzobre and Castroviejo 1993	Europe, America	S, W, SW and NW Europa	12*
L. pseudolaxiflora Lojac.	?	Bartolo et al. 1981	Africa, Europe	Europe: Madrid	12*
L. pseudoviscosa Murb.	*Versicolores*	http://www.rjb.csic.es/snapdragons, Viano 1975	Africa	N and C Africa	12*
L. purpurea (L.) Mill.	*Linaria*?	http://www.botanicalkeys.co.uk /flora, Colombo et al. 1978, Heitz 1926, Valdes 1970b, Fernandes et al. 1977	N Europe, America, Africa	S Europa	12*
L. ramosissima Wall.	?	Verma and Dhillon 1967	Asia	Asia	18**
L. reflexa Desf.	*Diffusae*	Goldblatt 1984	Africa, Europe, Asia	W Mediterranean	12*
L. repens (L.) Mill.	*Speciosae*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, http://www.botanicalkeys.co.uk/flora, Chater et al. 1972, Mateu and Mansanet 1982, Boscaiu et al. 2000, Heitz 1926, Valdés 1969; 1970b, Löve and Kjellqvist 1974, Fernandes et al. 1977, Laane and Lie 1985	N Europe, America, Asia	W, NW and C Europe	12*
L. reticulate Desf.	?	East 1933	Africa, America: Massachusetts	America: Massachusetts	12*
L. rubrifolia Robill and Cast. ex DC.	*Chaenorrhinum*	Cardona 1991	Africa, Europe	Mediterranean	14**
L. sagittata (Poir.) Steud., Syn. *Kickxia heterophylla* (Schousb) Dandy	?	Vilhelm Dalgaard 1986, Podlech 1986, Aldridge and Ortega 1976	Africa: Sahara, Europe: Canary Islands, Asia: Macaronesia, America	America: Massachusetts, Asia: Macaronesia, and SW, N Africa, Europe: Eastern	18**

				Canary Islands and Sahara	
L. salzmannii Boiss	*Versicolores*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Viano 1974, Chater et al. 1972	Europe	SW Europe	12*
L. saxatilis (L.) Hoffmanns. and Link	*Supinae*	http://www.rjb.csic.es/snapdragons, Chater et al. 1972, Baltisberger and Charpin 1989, Caixinhas et al. 1991, Heitz 1926, 1927a, East 1933, Valdés 1969, 1970b, Fernandes et al. 1977	Europe, Africa	C, N and SW Europe	12*
L. simplex Desf.	*Supinae*	http://www.rjb.csic.es/snapdragons, Chater et al. 1972, Snogerup 1985, Valdes 1969; 1970b; Ghaffari and Tajik 2007	SW Asia, Europe, Africa	S Europe, N Africa, SW Asia, S Russia	12*
L. spartea (L.) Hoffmanns. and Link	*Macrocentrum Versicolores*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Viano 1973; 1974, Heitz 1927a, Fernandes et al. 1977, Chater et al. 1972, Love and Love 1982, Diosdado et al. 1993	N Europe, America	C, SW Europe	12*
L. striatella Kuprian	*Linaria*	http://www.rjb.csic.es/snapdragons, Aryavand 1983	Asia	C. and SW Asia, Russia	12*
L. supina (L.) Chaz.	*Supinae*	http://www.rjb.csic.es/snapdragons, http://www.botanicalkeys.co.uk/flora, Heitz 1927a, Valdes 1973, Fernandez Casas et al. 1980a, Fernandez Casas and Perez-Chao 1978, Fernandes et al. 1977, Cardona 1977	Europe, Africa, SW Asia America	SW Europe	12*
L. tenuis Spreng.	*Versicolores*	Diaz Lifante et al. 1992	Africa, Asia:Middle East	Mediterranean: N Negev	12*
L. texana Scheele	?	Ward 1983	America	America: New Mexico and southern Colorado	12*
L. tingitana Boiss. and Reuter	?	http://www.rjb.csic.es/snapdragons, Humphries et al. 1978	Europe, Africa	SW Europe, NW Africa	12*
L. triornithophora (L.) Willd.	*Pelisseriana e*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Chater et al. 1972, Vazquez et al. 2001, Heitz 1926, Queiros and Santos 1977, Van Loon and Dejong 1978	Europe	SW Europe	12*
L. triphylla (L.) Miller	*Diffusae*	http://www.rjb.csic.es/snapdragons (2*n* = 12), http://www.floravascular.com (2*n* = 12), Chater et al. 1972 (2*n* = 12), East 1933(2*n* = 12, 14), Heitz 1926; 1927b (2*n* = 12), Valdes 1969; 1970b (2*n* = 12), Viano 1973; 1974 (2*n* = 12), Darlintong and Wylie 1955 (2*n* = 12, 14).	Europe, Africa, SW Asia, America: Massachusetts	America: Massachusetts, W and S Mediterranean, Asia: Russia	12* 14**
L. tristis. (L.) Mill.	*Supinae*	http://www.rjb.csic.es/snapdragons, Heitz 1926, 1927a, Chater et al. 1972, Galland 1988, Favarger et al. 1979, Vogt and Oberprieler 1994, Aparicio Martinez 1993, Valdés 1969	Europe, N Africa	NW Africa, S and SW Europe	12*
L. tursica valdes and Cabezudo	*Supinae*	http://www.rjb.csic.es/snapdragons, Valdes and Cabezudo 1977, Chater et al. 1972	Europe	SW Europe	12*
L. ventricosa Cosson and Bal.	*Speciosae*	http://www.rjb.csic.es/snapdragons, Fernandes et al. 1977, Humphries et al. 1978	N Africa, SW Europe	NW Africa	12*
L. verticillata Boiss.	*Supinae*	Liorenc Saez and Manuel Crespo 2004, Chater et al. 1972, Luque and Lifante 1991	Europe, Africa: (Nevada)	SW Europe, N Africa	12*
L. viscose Dum. Courset	*Versicolores*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, Chater et al. 1972, Löve and Kjellqvist 1974, Fernandes et al. 1977, Valdes 1969; 1970b, Viano 1979; 1973	Europe, Africa	SW Europe, NW Africa, Canaries Trigales	12*
L. vulgaris Miller	*Linaria*	http://www.rjb.csic.es/snapdragons, http://www.floravascular.com, http://www.botanicalkeys.co.uk/flora, Dmitrieva and Parfenov 1985, Dmitrieva 1986, Van Loon and Setten 1982, Laane et al. 1999, Van Den Brand and Wieffering 1979, Arohonka 1982, Subramanian and Pondmudi 1987, Probatova et al. 2001, Chuang and Heckard 1992, Dempsey et al. 1994, Montgomery et al. 1997, Javourkova-Jarolimova 1992, Mesicek 1992, East 1933, Chater et al. 1972, Heitz 1926; 1927a, Valdés 1969, Fernandes et al 1977, Van Loon and Van Setten 1982	S, C Europe,Australia, Asia: Far East, Alaska, Western Siberia, America	S, C Europe, SW Asia, Russia, Far East, N Africa	12*

It might be hypothesized that regions with highly diversified floras, such as the Iberian Peninsula, could be characterized by more polyploid species than the other regions. It seems that the natural hybridization is another significant force in plant evolution of this genus (Ranjbar et al. 2012, Ranjbar and Mahmoudi 2013a, 2013b, Ranjbar and Mahmoudian 2015, Ranjbar et al. 2014, Ranjbar et al. 2015). With a narrow scope (between *Linaria vulgaris* P. Mill. and *L. dalmatica* (L.) P. Mill in U.S. intermountain West (Ward et al., 2009), historical hybridization between closely related *Linaria* species, and the high number of species in the Mediterranean (104 spp.), suggests that this group is likely to have undergone a rapid diversification (Sutton, 1988). Nevertheless, spontaneous hybridization between *Linaria* species within the native Eurasian range of the genus has previously been reported. Considering the palaeogeography of this region and the potential broad-scale effects on gene regulation and developmental processes, the tertiary Iberian orogeny and the subsequent glaciations may have led to important changes in climate and topography (Thompson, 1999), which may have provided opportunities for the establishment of polyploids through hybridization and other mechanisms in the contact zones between existing species. Only about 1% of *Linaria* species represented n = 13 and 2n = 15 which may possess a hybrid origin among species which are available in the Mediterranean margin. There is *L. hellenica* (2n = 26) which is likely a hybrid between n = 6 and n = 7 or is an aneuploid (n = 12+1) from a tetraploid species with n = 12 (figure 5).

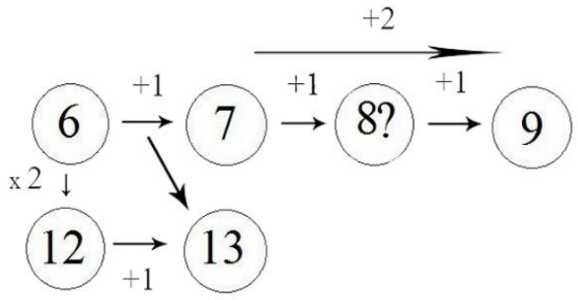

Figure 5. The proposed evolutionary pattern within the genus *Linaria* based on chromosome numbers.

Also *L. melanogramma* by Aryavand 1983 reported in Iran with 2n = 15 that the questioners can be a hybrid of n = 6 and n = 9 (*L. ramosissima*, 2n = 18 in Asia, is likely to occur in Bangladesh) (figure 5). The third factor of speciation by changes in the chromosome numbers is common in flowering plants and an almost characteristic occurrence in angiosperms. Aneuploidy and disploidy are the common phenomena in plants resulted from the loss or gain of one or a few numbers of chromosomes. According to Abou-el-Enain (2002), aneuploidy is an evolutionary process producing variations in plants. Although there are numerous examples of progressive loss of chromosomes in plants, the factors driving unemployed have been far less investigated than those of polyploidy. In *Linaria* genus these unemployed species are sporadically distributed among geographically disparate Old and New World species. A species with n = 7, 9 are both derived by descending aneuploidy from higher numbers and argues that n = 7, 9 could be produced by ascending aneuploidy. Origin of species with n = 7 which are annuals is likely n = 6 and origin of species with n = 9 which are perennials is likely n = 8 that possibly could not stand out through evolutionary process. Finding occasional plants in various populations with aneuploid chromosome numbers belonging to this genus indicates that aneuploid gametes not only are produced, but actually are functional. However the transmittance n = 6, 7 and 9 there is in all zones. No species having n = 13 are known from the New World and no species having n = 7 are known from Asia, even though this is the most common number in the New World and Mediterranean region. It is likely appeared that multiple origins of aneuploidy in *Linaria* have occurred, perhaps via non disjunction in n = 6 crosses, hybridization between n = 6 and n = 7 species, or aneuploid addition from n = 13, as in the case of the n = 12 species, so that species with n = 7 are derived through enabled loss. Due to the high detail it is appeared that in spite of marked morphological differentiation, species divergence within *Linaria* is relatively recent and reproductive isolation has not yet fully evolved (Ward et al., 2009). However, it can be concluded that the variation in chromosome number in the genus can be referred to the diversity in environmental constraints (Ranjbar et al., 2010b; 2011a). On the other hand, these variations represent a complex evolutionary pattern between the taxa (figure 5). Using flow cytometry, it has been possible to characterize the geographical distribution of cytotypes (within species or between closely related species), with the main advantage, in comparison with related techniques (e.g., Chromosome counting) and in a relatively short period of time. It seems that, to shed further light on the origin of polyploidy *Linaria* species, more complete cytogenetic and also molecular evidence will be necessary. In addition, more accessions should be cytogenetically studied and a directed

analysis through genomic (GISH) and fluorescent in situ hybridization (FISH) would supply interesting and important information.

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Research Article

Comparative Cytogenetic Analysis in the Populations of House Mouse Group, *Mus musculus* L.1766 (Cytotype 2n = 40) (Rodentia: Muridae) in Iran

Farahnaz Molavi [1], Jamshid Darvish [1,2]*, Farhang Haddad [1], Maryam M. Matin [1,3]

[1]*Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
[2]*Rodentology Research Department, Ferdowsi University of Mashhad, Mashhad, Iran*
[3]*Cell and Molecular Biotechnology Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran*

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Abstract

Cytotaxonomy is a branch of cytogenetics, devoted to the comparative study of karyological features for systematic and evolutionary purposes. Surely, awareness of chromosomal characters increases our knowledge in different fields of studies. In this study, cytogenetic analyses were performed in 92 *Mus musculus* specimens from 26 localities in Iran. Cytogenetic characteristics of the house mouse, *Mus musculus*, in Iran show that the chromosome number is 2n=40 and the arm number is NF=40. The karyotyping results indicated the presence of 20 Acrocentric (A) chromosome pairs. The L/S (r ratio) was between 2.0621 and 4.5862. The length of shortest chromosome, length of longest chromosome and mean of chromosomal length in different populations were between 2-3.58, 6.07-7.01 and 3.43-5.05 (µm), respectively. The results showed two distinct karyotypic formulae, namely cytotype B and cytotype C. Asymmetry indexes (AI, DI, As%, A, A2, A1 and Syi%) in all population except Birjand and Khash showed symmetry in chromosomes. In clustering methods using the matrix of symmetrical indexes similarities, four clusters were revealed, one for specimens of central and east of Iran, the second cluster for specimens from south and west of Iran, the third cluster was related to the eight specimens of Birjand and finally, the fourth cluster for two specimens of Khash locality.

***Keywords*:** Cytotaxonomy, Systematic, Chromosome structure, House mouse, Karyology, Iran, Middle East

Introduction

Now a day, nine species have been recognized in the genus Mus. This taxon arose within the last 4 Myr (Bonhomme and Guénet, 1996). Mus musculus was originally a Palearctic species, but now it has been spread throughout the world by humans and lives as a human commensal (Musser and Carleton, 2005). Genetic studies have revealed three peripheral geographic populations of house mouse as Mus musculus musculus, M. m. domesticus and M. m. castaneus (Vanlerberghe et al., 1986; Orth et al., 1996; Darvish et al., 2006; Rajabi-Maham et al., 2007). Cytotaxonomy is a branch of cytogenetics, devoted to the comparative study of karyological features for systematic and evolutionary purposes (Siljak-Yakovlev and Peruzzi, 2012). Today, a number of data can be obtained by chromosome studies including chromosome number, karyotype structure, karyotype asymmetry, chromosome banding, FISH (Fluorescence in situ hybridization), Genomic In Situ Hybridization (GISH) and chromosome painting (Graphodatsky et al., 2011; Cazaux et al., 2012). Among karyotype asymmetry is, one of the most popular, cheap and widely approaches which is used for determining of karyotype asymmetry (Peruzzi and Eroğlu, 2013). The concept of karyotype asymmetry, i.e. a karyotype marked by the predominance of chromosomes with terminal/subterminal centromeres (intrachromosomal asymmetry) and highly heterogeneous chromosome sizes (interchromosomal asymmetry), was developed for the first time by Levitsky (1931). Stebbins in 1971 proposed a quali-quantitative method for the estimation of karyotype asymmetry in twelve categories, by taking into account four classes (from 1 to 4), defined according to the increasing proportion of chromosomes with arm ratio < 2:1, to be combined with three classes (from A to C) defined according to the increasing ratio between

Corresponding authors E-mail:
* Darvish_j2001@yahoo.com

largest and smallest chromosomes in a complement. Concerning interchromosomal asymmetry, which is due to heterogeneity among chromosome sizes in a complement, other researchers proposed quantitative estimation methods in the subsequent years. This is the case of the Rec index (Greilhuber and Speta, 1976; Venora *et al*., 2002), the A2 index (Romero Zarco, 1986), the R ratio (Siljak-Yakovlev, 1996) and the CVCL (Lavania and Srivastava, 1992; Watanabe *et al.*, 1999; Paszko, 2006). The latter, actually a coefficient of variation, is a statistically correct parameter and is able to capture even small variations among chromosome sizes in a complement (Peruzzi and Eroğlu, 2013).
Cytological investigations have shown that the basic chromosome number is constant among the *Mus* species. Despite this stability in chromosome number, large variations in chromosome size have played a major role in the evolution of some species (Guillermoseijo and Fernandez, 2003; Peruzzi and Eroğlu, 2013). Many molecular studies have been performed on house mouse (Rajabi-Mahan *et al*., 2007; Darvish *et al*., 2006), but there is a paucity of data for chromosomal morphological characters. From the available information, we do not have any claim of morphological uniformity of chromosomes and homogeneous karyotype arrangements. From a karyosystematic point of view, until now, no group has been classified according to their karyotype morphology. Thus, in this study the karyotypes of specimens captured from 26 localities are analyzed with the following objectives: (1) to clarify the morphology of chromosomes in house mouse of Iran, (2) to examine the patterns of chromosome variations in populations of this taxon.

Materials and Methods

During field excursions in 26 stations of Iran, 92 rodent species were captured (Fig. 1 and Table 1).
Mice were caught using Longworth live-traps in farm buildings. Four morphometric characters including the length of body, tail, ear and hind foot were measured and the animals were karyotyped according to the conventional bone marrow method. The zygomatic index (ZI = width of molar process / width of upper part of zygomatic arch) and the ratio between tail length to head and body length (tail length / head body length = T / HB) were determined to identify *Mus* species. Voucher specimens were skinned and stuffed in the standard museum manner. The skin, skulls and karyotype preparations were deposited to Rodentology Research Department, Ferdowsi University of Mashhad.

Mitotic chromosome preparations were made with a modification of the technique described by Summer (1972). To do so, 1 ml of colchicine solution (0.25 mg/ml) per hundred grams of body weight was injected intraperitoneally to each specimen. One hour after colchicine injection, animals were anesthetized with Ether. Bone marrow was separately transferred to a small watch glass containing 8-10 ml hypotonic solution (0.085 M KCl) at 37°C, lasting time for hypotonization treatment was 20-25 min.

Fixation

The swollen cell suspensions were fixed in 3:1 cooled Carnoy's fixator (3:1 methanol/acetic acid glacial) for 20 min, then the old fixative was replaced with the fresh solution and repeated for three times.

Slide preparation

The slides were prepared by dropping two drops of the fixing solution containing the cell suspension onto the clean slides from 60 cm height. The slides were stained in a 5% Giemsa (Merck) for ten min.
At least 10 metaphases were analyzed for each population (including 2–8 individuals) using a ax100 zoom digital CCD camera, selecting the five best for measurements. Fourteen karyological characteristics (Table 2) of all specimens were prepared by Karyological Analysis software (version 1.2, 2010) and CIP Software. The relative length of each pair was expressed by the percentage of the absolute length of each chromosome pair divided by the sum of the absolute length of total chromosomes.
The chromosome pairs were classified according to Levan *et al.* (1964). The pair numbers were definitely attributed following this classification and in decreasing length order within each class. Means were compared by one-way ANOVA after Bartlett's test of homogeneity. Also, Tukey's test was carried out to measure differences between each pair of means. A cluster analysis of the karyotypic data was carried out to examine karyotype similarities among populations. A data matrix of 26 OTUs (operational taxonomic units) including 14 variables was constructed. Statistical analysis was performed using the SPSS program, version16,0 (2011). Clustering was performed using the unweighted pair-group method (UPGMA).

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Figure 1. Collecting localities for M. musculus analyzed in this study (black boxes are stations)

Table1. The coordinates of sampling localities

	Taxon	Localities	Latitude	Longitude
1	*M. m. musculus*	Kakhk	34° 8′ 59″N	58° 38′ 21″ E
2	*M. m. musculus*	Gonabad	34° 21′ 10″ N	58° 41′ 1″ E
3	*M. m. musculus*	Torbate Jam	35° 14′ 38″ N	60° 37′ 21″ E
4	*M. m. musculus*	Sarakhs	36° 32′ 42″ N	61° 9′ 28″ E
5	*M. m. musculus*	Dargaz	37° 26′ 40″ N	59° 6′ 29″ E
6	*M. m. musculus*	Kalat	36° 59′ 33.01″ N	56° 45′ 23.83″ E
7	*M. m. castaneus*	Isfahan	32° 63′ 35″ N	51° 65′ 36″ E
8	*M. m. castaneus*	Zahedan	29° 29′ 47″ N	60° 51′ 46″ E
9	*M. m. castaneus*	Khash	28° 13′ 16″ N	61° 12′ 57″ E
10	*M. m. castaneus*	Zabol	31° 1′ 43″ N	61° 49′ 4″ E
11	*M.m. domesticus*	Chabahar	25° 17′ 31″ N	60° 64′ 35″ E
12	*M. m. castaneus*	Shiraz	29° 61′ 0″ N	52° 54′ 0″ E
13	*M. m. castaneus*	Yazd	31° 53′ 50″ N	54° 22′ 4″ E
14	*M. m. musculus*	Esfarayen	37° 73′ 03″ N	57° 50′ 72″ E
15	*M. m. domesticus*	Eizeh	31° 50′ 48″ N	49° 50′ 36″ E
16	*M. m. castaneus*	Mamasani	30° 7′ 0″ N	51° 31′ 0″ E
17	*M.m. domesticus*	Uromia	37° 33′ 19″ N	45° 4′ 21″ E
18	*M. m. domesticus*	Khalkhal	37° 37′ 8″ N	48° 31′ 33″ E
19	*M. m. musculus*	Mashhad	36° 18′ 0″N	59° 36′ 0″ E
20	*M. m. domesticus*	Zanjan	36° 40′ 0″ N	48° 29′ 0″ E
21	*M. m. musculus*	Birjand	32° 87′ 0″ N	59° 20′ 0″ E
22	*M. m. domesticus*	Ahvaz	36° 40′ 0″ N	48° 29′ 0″ E
23	*M. m. castaneus*	Qasr-e Qand	26° 14′ 54″ N	60° 45′ 9″ E
24	*M. m. castaneus*	Rask	26° 14′ 13″ N	61° 23′ 56″ E
25	*M. m. castaneus*	Kerman	30° 17′ 0″ N	57° 5′ 0″ E
26	*M. m. domesticus*	Mahshahr	30° 54′ 32″ N	49° 11′ 58″ E

Table 2. List of characters used for chromosome analysis

	Name	Definition	Formula	Range	Reference	Description
1	2n	Diploid number of chromosomes	sum of chromosomes	>2	Nägeli , 1842	-
2	Fn	Fundamental number	number of visible major chromosomal arms per set of chromosomes	Fn ≤ 2 x 2n	Matthey, 1945	-
3	Fna or An	Autosomal fundamental number	number of visible major chromosomal arms per set of autosomes (non-sex-linked chromosomes).	Fna≤ 2 x 2n	(Matthey, 1945)	-
5	A1	The intra chromosomal asymmetry index	$(\sum_{i}^{n} qi/pi)/n$	0-1	Romero Zarco, 1986	P: long arm, q: short arm, and n: total of chromosome
6	A2	The inter chromosomal asymmetry index	Scl/Xcl	0-1	Romero Zarco, 1986	Scl:Standard error of total chromosomal length. Xcl: Mean of total chromosomes
7	A	The degree of asymmetry of karyotype	$\frac{\sum_{i=1}^{n} \frac{pi-qi}{pi+qi}}{n}$	0-1	Watanabe et al., 1999	P:long arm, q: short arm, n:total of chromosome
8	DI	The dispersion index(is a normalized measure of the dispersion of a probability distribution)	$D= \sigma^2/\mu$	>0	Lavania and Srivastava, 1992	σ^2: variance μ,: mean
9	AI	The asymmetry index	$(\mu_x - \mu_y V)/(\sigma x^2 + \sigma y^2 V^2)^{1/2}$	0 < x ≤ 2.0: The asymmetry is weak. The distribution is relatively symmetr ical. 2.0 < x ≤ 4.0: The asymmetry is moderate. The distribution is relatively asymme trical.	Paszko, 2006	$V = (R - L)/(R + L) \equiv X/Y$; X:= $R - L$ and Y : $R + L$ μ_x: Means X μ_y : Means Y σx^2: variances

				x > 4.0: The asymmetry is strong. The distribution is asymmetrical.		
10	Cytotype	An individual of a species that has a different chromosomal f actor to another (e.g. haploid versus diploid)	-	-	-	-
11	L/S	arm ratio(r)	long arm/short arm	-	-	-
12	As%	The karyotype asymmetry index	(Length of long arm in chromosome complements/Total sum of chromosome length in a set)x100	50-100	Arano, 1963	-
9	TF%	The total form percent	Total of short chromosomal lengths/ Total of chromosomal lengths	0-50	Huziwara, 1962	-
10	Syi%	The index of karyotype symmetry	$\frac{Ms}{Ml}$	-	Greilhuber and Speta, 1976, Venora et al. 2002	Ms:Mean length of the shortarms Ml:Mean length of long arms
11	Rec	The index of chromosomal size resemblance	$= ((\sum_{i=1}^{n} CLi/LC)/ n) * 100$ i=1-40, n=40	0-100	Greilhuber and Speta, 1976, Venora et al. 2002	CLi: Length of total of chromosome LC: Length of longest chromosome
12	SC	Length of shortest Chromosome	micron	-	-	-
13	LC	Length of longest Chromosome	micron	-	-	-
14	Mpq	Mean of chromosomal Length	micron	-	-	-

Result:

Analyzing chromosome numbers of all specimens from 26 localities showed that they all had 40 chromosomes (2n=40). This result was observed for over 90% of cells in metaphase. Observation of cells lacking a normal number of chromosome (2n=36-38) was probably due to chromosome losses during preparation or mixing with nearby cells. All chromosomes in prepared karyotypes had a homologous pair. Homologous pairs of chromosomes were arranged according to size decrease and centromeric indexes. The Y chromosomes could be distinguished by small size

and dark color. The representative karyotypes for *M. musculus* captured from different localities are shown in Fig 2.

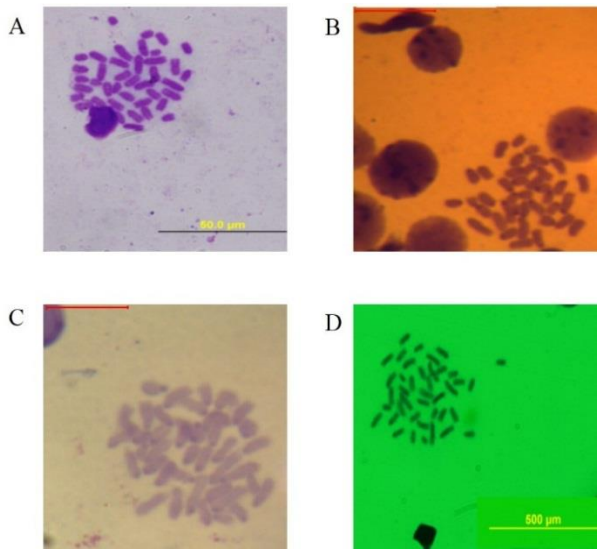
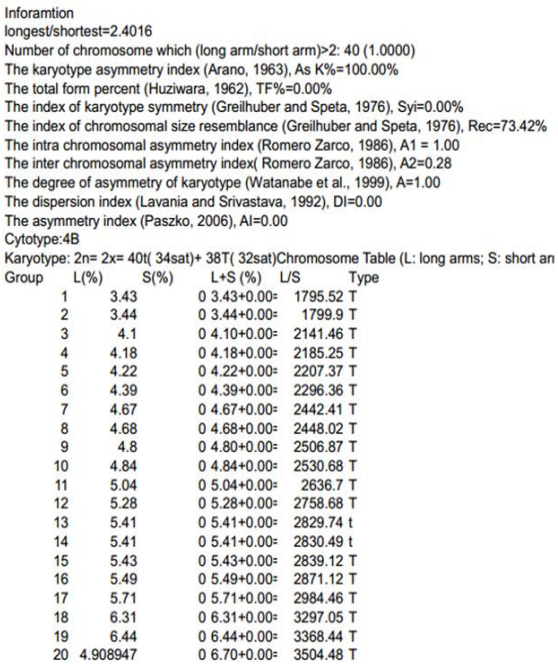


Figure 2. Chromosome spreads of Mus musculus, all specimens with 2n=40. Animals were captured from A: Mashhad, B: Chabahar, C: Uromia and D: Mahshahr



Inforamtion
longest/shortest=2.4016
Number of chromosome which (long arm/short arm)>2: 40 (1.0000)
The karyotype asymmetry index (Arano, 1963), As K%=100.00%
The total form percent (Huziwara, 1962), TF%=0.00%
The index of karyotype symmetry (Greilhuber and Speta, 1976), Syi=0.00%
The index of chromosomal size resemblance (Greilhuber and Speta, 1976), Rec=73.42%
The intra chromosomal asymmetry index (Romero Zarco, 1986), A1 = 1.00
The inter chromosomal asymmetry index(Romero Zarco, 1986), A2=0.28
The degree of asymmetry of karyotype (Watanabe et al., 1999), A=1.00
The dispersion index (Lavania and Srivastava, 1992), DI=0.00
The asymmetry index (Paszko, 2006), AI=0.00
Cytotype:4B
Karyotype: 2n= 2x= 40t(34sat)+ 38T(32sat)Chromosome Table (L: long arms; S: short an

Group	L(%)	S(%)	L+S (%)	L/S	Type
1	3.43	0	3.43+0.00=	1795.52	T
2	3.44	0	3.44+0.00=	1799.9	T
3	4.1	0	4.10+0.00=	2141.46	T
4	4.18	0	4.18+0.00=	2185.25	T
5	4.22	0	4.22+0.00=	2207.37	T
6	4.39	0	4.39+0.00=	2296.36	T
7	4.67	0	4.67+0.00=	2442.41	T
8	4.68	0	4.68+0.00=	2448.02	T
9	4.8	0	4.80+0.00=	2506.87	T
10	4.84	0	4.84+0.00=	2530.68	T
11	5.04	0	5.04+0.00=	2636.7	T
12	5.28	0	5.28+0.00=	2758.68	T
13	5.41	0	5.41+0.00=	2829.74	t
14	5.41	0	5.41+0.00=	2830.49	t
15	5.43	0	5.43+0.00=	2839.12	T
16	5.49	0	5.49+0.00=	2871.12	T
17	5.71	0	5.71+0.00=	2984.46	T
18	6.31	0	6.31+0.00=	3297.05	T
19	6.44	0	6.44+0.00=	3368.44	T
20	4.908947	0	6.70+0.00=	3504.48	T

Figure 3. An example of the output in karyotype analysis software, this output belongs to a speciemen from Torbat-Jam.

Fourty acrocentric chromosome pairs were observed in all preparations. The number of chromosome arms was determined as NF=40. All chromosomal criteria were calculated for each specimen in karyotype analysis software separately (Fig. 3). All results are summarized in Table 3.

The L/S index was between 2.0621 to 4.5862. The length of shortest chromosome, length of longest chromosome and mean of chromosomal length in different populations were between 2-3.58 , 6.07-7.01 and 3.43- 5.05 (µm), respectively The UPGMA dendrogram was constructed on the. basis of seven symmetrical indexes (Fig. 4).

Table 3. Comparison of karyological records of the given house mouse populations.

	Localities	2n	Fn	Fna	L/S	AsK%	TF%	Syi%	Rec	A1	A2	A	DI	AI	Cytotype	SC	LC	Mpq
1	Torbat Jam	40	38	38	2.8071	100	0	0	64.76	1.00	0.00	0.26	0	0.00	4B	3.58	6.42	3.96
2	Kakhk	40	38	38	2.4739	100	0	0	65.01	1.00	0.00	0.27	0	0.00	4B	3.48	6.41	4
3	Dargaz	40	38	38	2.9631	100	0	0	65.75	1.00	0.00	0.27	0	0.00	4B	3.58	6.42	4.09
4	Sarakhs	40	38	38	3.0152	100	0	0	64.69	1.00	0.00	0.28	0	0.00	4B	3.31	6.91	4.03
5	Mashhad	40	38	38	2.5987	100	0	0	64.98	1.00	0.00	0.22	0	0.00	4B	3.21	6.81	4.28
6	Esfarayen	40	38	38	2.4021	100	0	0	73.42	1.00	0.00	0.28	0	0.00	4B	3.43	6.07	3.43
7	Gonabad	40	38	38	3.6131	100	0	0	60.68	1.00	0.00	0.29	0	0.00	4B	3.11	7.01	4.23
8	Birjand	40	38	38	3.6289	97.38	2.62	2.69	47.65	0.97	0.29	0.96	0	0.77	3C	3.02	6.88	4,767
9	Kalat	40	38	38	2.7846	100	0	0	64	1.00	0.00	0.28	0	0.00	4B	3.19	6.71	4
10	Shiraz	40	38	38	2.7475	100	0	0	65.27	1.00	0.00	0.23	0	0.00	4B	3.54	6.62	4.98
11	Mammasani	40	38	38	2.7475	100	0	0	65.27	1.00	0.00	0.22	0	0.00	4B	3.14	6.54	4.9
12	Eizeh	40	38	38	2.6378	100	0	0	61.2	1.00	0.00	22.00	0	0.00	4B	3.15	6.59	495
13	Isfehan	40	38	38	2.6998	100	0	0	66.11	1.00	0.00	0.27	0	0.00	4B	3.21	6.46	4.97
14	Yazd	40	38	38	2.6521	100	0	0	68.21	1.00	0.00	0.22	0	0.00	4B	3.34	6.37	5.01
15	Kerman	40	38	38	2.6435	100	0	0	66.31	1.00	0.00	0.24	0	0.00	4B	3.29	6.57	4.99
16	Rask	40	38	38	2.6415	100	0	0	68.87	1.00	0.21	0.21	0	0.00	4B	3.57	6.55	5
17	Qasr-e Qand	40	38	38	3.1735	100	0	0	61.68	1.00	0.00	0.26	0	0.00	4B	3.13	6.37	4.92
18	Zahedan	40	38	38	2.6875	100	0	0	62.05	1.00	0.00	0.25	0	0.00	4B	3.34	6.43	4.96
19	Zadul	40	38	38	2.6576	100	0	0	65.43	1.00	0.00	0.25	0	0.00	4B	3.45	6.54	4.88
20	Chahbahar	40	38	38	2.2314	100	0	0	65.48	1.00	0.00	1.00	0	0.00	4B	2.67	6.07	4.47
21	Kalkhal	40	38	38	2.1754	100	0	0	64.87	1.00	0.00	1.00	0	0.00	4B	2.69	6.1	4.38
22	Mahshahr	40	38	38	2.0921	100	0	0	63.26	1.00	0.00	1.00	0	0.00	4B	2.03	6.08	4.59
23	Khash	40	38	38	4.5862	90.95	9.05	0.95	42.32	0.91	0.35	0.87	0	4.21	3C	2.98	6.12	4.46
24	Uromia	40	38	38	2.1756	100	0	0	68.07	1.00	0.00	0.27	0	0.00	3C	2.45	6.4	5.05
25	Zanjan	40	38	38	2.2414	100	0	0	65.43	1.00	0.00	1.00	0	0.00	4B	2.69	6.05	4.45
26	Ahvaz	40	38	38	2.0621	100	0	0	61.26	1.00	0.00	1.00	0	0.00	4B	2	6.06	4.53

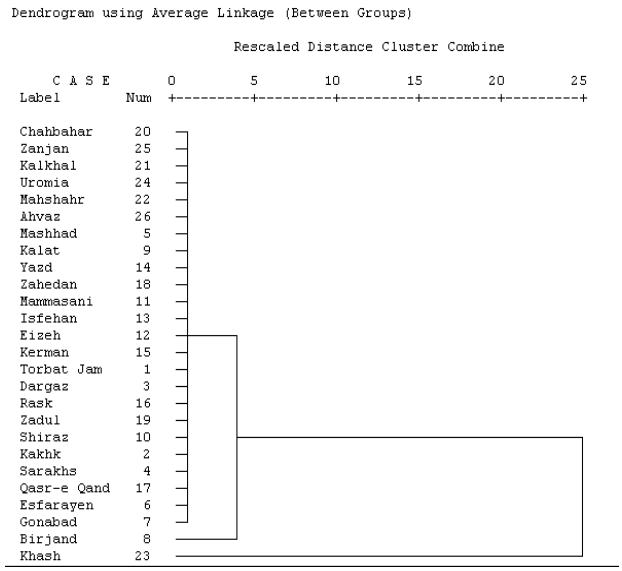


Figure 4. Dendrogram showing the phonetic relationship among the studied localities of *Mus musculus*, constructed using the matrix of symmetrical indexes similarities with UPGMA.

In general, karyotypes were symmetric, except eight specimens from Birjand and two specimens from Khash. The UPGMA dendrogram constructed on the basis of karyotype similarities (Fig. 5) showed four major clusters. The first cluster is comprised of speciemens captured from south and west of Iran, characterized by the smallest size of their chromosomes.

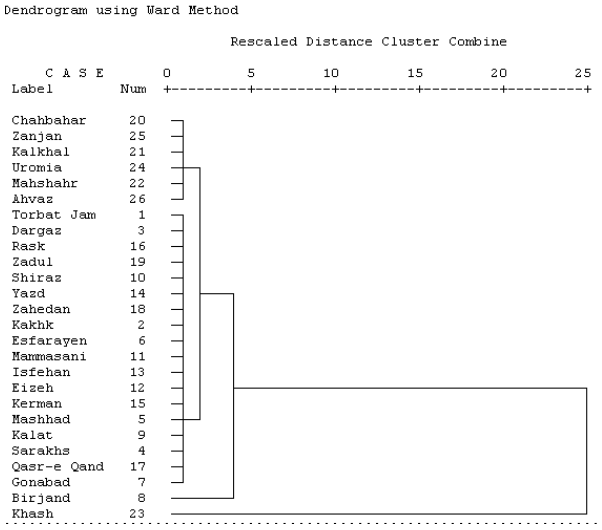


Figure 5. Dendrogram showing the phonetic relationships among the populations of *Mus musculus* captured from different localities, constructed using the matrix of karyotype similarities with UPGMA

All specimens from central and eastern Iranian house mouse are separated by the greater phenetic distance and they are placed in second cluster.

The third and fourth clusters contain eight samples from Birjand and two samples from Khash, respectively, which are characterized by asymmetry in their symmetry indexes and their different cytotype. Sizes of chromosomes in Birjand speciemens are more similar to eastern and central specimens and sizes of chromosomes in specimens from Khash are more similar to western and southern specimens of Iran.

In summary, on the basis of the morphology of their chromosome complement and quantitative parameters, two main groups of karyotypes can be distinguished in this study: one of them is related to populations living in south and west of Iran and the other is related to mice captured from the east and central parts of Iran.

Discussion

Mus musculus is usually stable from karyotypic point of view, with little or no variation in diploid number and chromosomal morphology. Standard karyotype of the house mouse is 2n=40, NF=40 and NFa=38 (Baydemir and Karoz, 2014). However, variations in the chromosome numbers have been reported in *Mus musculus domesticus*, for example, in Chile (Berríos, 2010), Turkey (G.zcelioÛlu *et al.*, 2005; Gündüz 2000 and Yigi, 2006) and Thailand (Badenhors, 2009). Karyotype formula and quantitative analyses have a great uniformity among populations of this species, except populations of *Mus musculus domesticus.* These studies support the hypothesis that claims intraspecific stability of karyotypes in house mouse subspecies (Baydemir and Karoz, 2014).

N Our results indicate that the chromosome numbers of species captured from Iran are the same with those published previously (Mirabzadeh, 2001; Silver, 2001). Although the diploid chromosome number is considered as 2n=40 in this study, several incomplete metaphases were also encountered in these preparations. The differences observed in the number and types of chromosomes in different studies may have various reasons. For example, differences in kind of techniques may lead to differences in the type, number of chromosome arms and even number of chromosomes. High concentrations or long treatment period of colchicine could also be the cause of conflict as concentrations or treatment periods of non-compliance can lead to a shortening of chromosomes and therefore the difference between the measurements and the arms of the chromosomes. According to Matthey (1954), Nadler and Lay (1967) and Vorontsov and

Korobitsina (1970), some species in this genus have different FN (Fundamental number). Our findings are not different for the FN or Fna (Autosomal fundamental number) of Iranian house mouse, and do not support the idea that there is a chromosomal variation in the FN of this species. Rutty, 1772, Orsini *et al.* (1983), Auffray *et al.* (1990) and Cucchi *et al.*, (2005) reported the same conclusion for the *Mus* species in Turkey.

Analysis of karyotypes showed that in general, the chromosomes were acrocentric (Nanda *et al.*, 1995; Manna, 1974) and of similar size (Goleman, 1996; Padilla-Nash, 2006) in all mice. They formed a homogeneous group and differed mainly in the length of the Y chromosome (Levan , 1962; Nesbitt and Francke, 1973). The X chromosome, which is one of the longest chromosomes could be easily detected (Cowell, 1984; Levan, 1962; Mirabzadeh, 2001). The Y chromosome was dark and the centromeric chromatin was not obvious (Cowell, 1984).

Chromosomes and evolution— Differences in karyotype formulae and asymmetry indexes found among species of different locations suggest that structural changes may have contributed to the diversification of the genus. On the other hand, the fact that species formed groups that share major karyotype characteristics may indicate that if the mechanisms of speciation within each group involved chromosome rearrangements, these may not include structural mutations, but small or cryptic changes. Alternatively, if speciation has occurred as a consequence of large chromosomal modifications, these may have been changes that did not modify the karyotype morphology, such as paracentric inversions or reciprocal translocations with segments of the equal size (Guillermoseijo and Fernandez, 2003). The existence of a similar karyotype in some species suggests that chromosome evolution in this section may be constrained to non-random changes with particular restrictions for the occurrence or fixation of structural rearrangements. The stability of complements among a group of species was first explained by ortho selection, which considers the occurrence of random chromosome mutations, but with the fixation of a restricted type of rearrangement (White, 1978). An alternative hypothesis was offered by King (1993), who considered the non-random nature of chromosomal evolution. This model contemplates that structural characteristics of the genome restricts the position and number of breaks that could occur and the type of rearrangements that could form. Even though both mechanisms would have similar results, a bulk of molecular and chromosome data is accumulating in favour of the position that claims that chromosomal mutations are not only non-random but are constrained by the chromosome structure to the type of change that can be produced (Peters, 1982; Shaw *et al.*, 1983; King, 1993; Narayan, 1988). Guillermoseijo and Fernandez (2003) showed that when the size of chromosomes varies without significant changes in karyotype formula, those changes in genome size may have been non-random and that the variations in DNA amounts are equally distributed among all chromosomes of the complements. Moreover, Seijo (2002) showed that the data obtained from banding patterns also support the non-randomness of genomic changes in some species because bands with similar base composition tend to have equilocal disposition in the karyotypes.

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Letter to Editor

CXCR4: A Potential Chemokine Receptor for Future Regenerative Therapeutic Target

Asieh Heirani-Tabasi and Hojjat Naderi-Meshkin*

Stem Cells and Regenerative Medicine Research Department, ACECR- Khorasan Razavi Branch, Mashhad, Iran

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Summary

Understanding the molecular mechanisms in regeneration could help the stem cell therapists to improve the clinical practices and could be considered a major milestone in the translation of stem cell research to clinics. Chemokines have been described as one of the most significant singling networks in stem cells homing and regeneration of damaged organs. CXCR4/CXCL12 has been known as a key player in this regard. Expression of CXCR4 has been observed in a number of cells such as mesenchymal stem cells, epithelial cells etc and plays crucial and unique role in the migration of cells towards a cytokine gradient and regulating stem cells trafficking as well as tissue/organ regeneration and embryogenesis.

***Keywords*:** Chemokine, CXCR4, Stem cell therapy, Regenerative Medicine

Dear Editor…

Damaging of tissues and organs by traumatic injuries and various diseases is a current big challenge for clinical researchers and practitioners to deal with it. Considerable developments in regenerative medicine have been seen in last decade as the researchers focused their attentions toward regenerative protocols for lost or damaged organ (Mao and Mooney, 2015).

A number of signaling pathways are involved in regeneration and development among which CXCR4/CXCL12 has been described as a key pathway in leukocyte trafficking, angiogenesis, inflammatory disorders, cancer and HIV pathology. Interaction between CXCR4 and its chemokine CXCL12 induces different downstream signaling such as cell survival, proliferation and chemotaxis (Vidaković et al., 2015). Therefore, due to the significance of CXCR4, targeting this cytokine for clinical research could create a hope for regeneration of damaged tissues. In contrast to other chemokine receptors, CXCR4 primarily functions in the immunity and regulation of inflammation through its expression in leukocytes (Hauser et al., 2002).

CXCR4 is also expressed in a variety of mesenchymal stem cells and epithelial tissues and plays crucial and unique role in the migration of cells towards a cytokine gradient and regulating stem cells trafficking as well as tissue/organ regeneration and embryogenesis (Naderi-Meshkin et al., 2015a). Stem cells have been described as the key agents of regenerative therapies but their escape after transplantation is a continuous challenge being faced. Stem cell homing is a multistage process similar to the leukocytes migration towards injury sites (Kucia et al., 2004). CXCR4/CXCL12 pathway plays role in the repair of different damages by promoting the migrational stem cells, such as attracting stem cells to the burnt injury sites and injured liver, accelerating wound healing to skin injury sites, repairing infarcted hearts and promoting repair of the injured kidney (Ghieh et al., 2015; Li et al., 2015a; Li et al., 2015b; Ling et al., 2016). It has been described that CXCR4 expression and cells homing to repair damaged tissue decreased following culture (Naderi-Meshkin et al., 2015b).

Dear editor, so far various therapeutic strategies such as treatment with chemical compounds, hypoxia, cytokines, growth factors and genetic modifications have been used to enhance the expression of CXCR4 to get enhanced efficacy in stem cells homing (Naderi-Meshkin et al., 2015a). Stem cell therapy has attracted researchers around the world, and a huge work has been done in this regard, however, but there is very rare clinical trials

*Corresponding author E-mail:
Hojjat_naderi@jdm.ac.ir

investigating the role of CXCR4 in tissue regeneration focusing its role in stem cells recruitment to the sites of injury and their maintenance. Considering the current reports mentioned in this letter, it could be concluded that CXCR4 is going to be the next therapeutic target but its efficiency and safety in regenerative therapy, has not been approved due to the lack of clinical data. Therefore, focusing CXCR4 in clinical trials of regenerative therapies will open the windows of possibilities to replace lost or damaged organs

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