Ferdowsi University of Mashhad
ISSN 2008-9147
Numbers: 11

JCMR

Journal of Cell and Molecular Research

Volume 6 , Number 1, Summer 2014

JCMR

بسم الله الرحمن الرحيم

Issuance License No. 124/902-27.05.2008 from Ministry of Culture and Islamic Guidance
Scientific Research Issuance License No. 161675 from the Ministry of Science, Research and Technology, Iran

Journal of Cell and Molecular Research (JCMR)

Volume 6, Number 1, Summer 2014

JCMR Office: Department of Biology, Faculty of Sciences, Ferdowsi University of Mashhad, Mashhad, Iran.
Postal Code: 9177948953
P.O. Box: 917751436
Tel./Fax: +98-513-8795162
E-mail: jcmr@um.ac.ir
Online Submission: http://jm.um.ac.ir/index.php/biology

Table of Contents

The Editorial

First five years of scientific publications in JCMR / The Editorial

Muhammad Irfan-Maqsood

JCMR Office, Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran
Editorial email: jcmr@um.ac.ir

Abstract

Journal of **C**ell and **M**olecular **R**esearch (**JCMR**) was first published in 2008 as the first journal in the field of cell and molecular biology research in Iran. The need for the establishment of JCMR was felt as a platform for the young cell and molecular biology researchers under the supervision of respective experts. JCMR was established to focus on almost all fields of cell and molecular research and some categorical publications were planned as focusing areas of modern and innovative research done by the young researchers. **Genetics**, **Bioinformatics**, **Biotechnology**, **Cell Biology** and **Molecular Biology** were remained the focusing areas of publications in the first five years of JCMR. The editorial board has proposed new strategies to increase the impact of this journal including focused and high impact publications and to fasten the peer-review processing of journal. Its indexing in international indexing databases like Scopus etc. will encourage the authors and editors as a milestone in the boosting of scientific quality data production.

***Keywords*:** Journal of cell and molecular research, ISSN 2008-9147, Ferdowsi University of Mashhad

Introduction

JCMR-Journal of Cell and Molecular Research was established in 2008 and published its first volume and issue in the beginning of 2009. The aim of this journal was to fulfill the need of a comprehensive journal covering almost all fields of cellular and molecular research in Iran. The published articles since then have covered many areas of cell and molecular research including Genetics, Bioinformatics, Biotechnology, Cell Biology and Molecular Biology. JCMR published research articles only, till 2013. After this, the editorial, commentaries, letters and reviews were also included in its categories of articles to highlight critical issues in cell and molecular research (Irfan-Maqsood and Hemmati-Sadeghi, 2013, Matin, 2014). In this editorial, we have discussed the citation metrics of and multi-disciplinary scope of JCMR in past five years.

Citation Metrics of JCMR:

JCMR is categorized as English language scientific research journal according to the Ministry of Sciences, Research and Technology Iran. Its first issue was published as Ferdowsi University International Journal of Biological Sciences which later on was renamed as Journal of Cell and Molecular Research in the same year. In 2009, upon its first publication, the JCMR received no any citation and remained uncited for the next year, 2010. From 2009 till last of 2010, JCMR published 29 research articles which got a total of 9 citations in 2011. It's increased indexing in different international indexing databases, its citation continued to be increased. From 2009-2013, JCMR has completed its first five years of publications. In these first five years, JCMR published 70 articles which have received a total of 52 citations calculating the average impact of JCMR as 0.7 citations/articles as shown in table 1.

Table 1: JCMR Citation metrics for first five years (2009-2013.

Year	No of Papers	No of Citations	Average Citations / Impact Factor (Annual)
2009	15	--	--
2010	14	--	--
2011	12	9	0.75
2012	13	11	0.84
2013	16	20	1.25
2014	--	9	--
Total	70	49	0.7

∗Corresponding author E-mail:
muhammadirfanmaqsood@gmail.com

Multidisciplinary scope of JCMR- Journal of Cell and Molecular Research:

JCMR selected, reviewed and published articles regarding selected fields of cell and molecular research i.e. Genetics, Bioinformatics, Biotechnology, Cell Biology and Molecular Biology. Sub-disciplines surrounding its scope have been categories as gene expression and marking (Beihaghi, 2009, Sepahi et al, 2013), molecular pathology, molecular informatics (Sadeghi, et al., 2012), molecular interactions of protein and nucleic acid (Raeisi, 2013), recombinant DNA technology, production of transgenic plants and animals, stem cell research (Mahmoudi, et al., 2011) and its clinical potential, cytological studies (Dowom et al., 2012), disease and metabolic studies and molecular mechanics of different small molecules and growth factors (Ghazvini, et al., 2009).

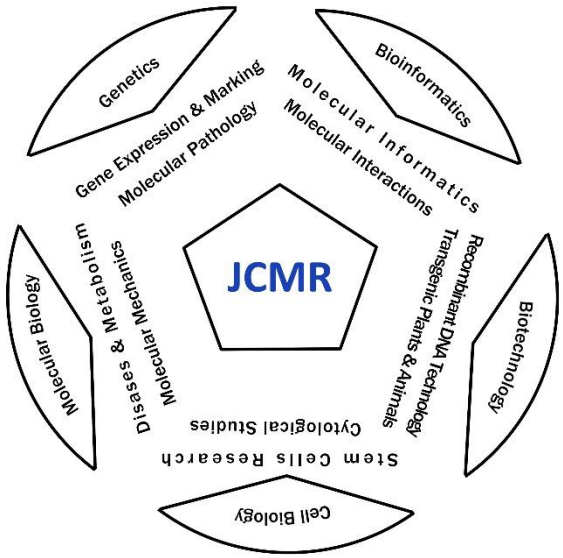

Figure 1: Disciplinary scope of JCMR-Journal of Cell and Molecular Research

Acknowledgement:

JCMR editorial board highly thank to the office of the Vice President for research and technology of the Ferdowsi University of Mashhad for their financial and administrative support to proceed the JCMR for editorial, reviewer, publication, printing and distribution processes. We also thank to our honorable scientific reviewers who helped us to make JCMR a prominent scientific and research journal in Iran.

References:

1. Matin M. M. (2013) Cancer, A Big Monster, Which Should Be Defeated / The Editorial. Journal of Cell and Molecular Research 5(2): 47.
2. Irfan-Maqsood M. and Hemmati-Sadeghi S. (2013) Developments toward an Ideal Skin Substitute: A Commentary. Journal of Cell and Molecular Research 5(2): 88-92.
3. Mohsenzadeh S., Najafi L., Amirghofran Z., Khosravi, A. R. (2009) Cytotoxic activity of Isatis campylocarpa, an Iranian endemic plant on human cancer cell lines. Journal of Cell and Molecular Research 1(1): 51-55.
4. Dowom S. A., Bahrami A. R., Abrishamchi P., Saeinasab M. (2011) Cytotoxic effect of essential oils from Salvia leriifolia Benth on human Transitional Cell Carcinoma (TCC) and mouse fibroblast. Journal of Cell and Molecular Research 3(2): 51-56.
5. Mahmoudi Z., Matin M. M., Saeinasab M., Nakhaei-Rad S., Mirahmadi M., Mahdavi-Shahri N., Mahmoudi M., Bahrami A. R. (2011) Blastema cells derived from rabbit ear show stem cell characteristics. Journal of Cell and Molecular Research 3(1): 25-31.
6. Ghazvini K., MirzaHesabi E., Akbarein M. M. (2009) Antibacterial activity of a malodor neutralizer containing silver nanoparticles. Journal of Cell and Molecular Research 1(1): 47-50.
7. Beihaghi M., Bahrami A. R. Bagheri A., Leegood R., Ghabooli M., Zolala J., Shahriari F. (2009) Comparison of pepck gene expression in developing seeds. Journal of Cell and Molecular Research 1(2): 61-67.
8. Sepahi S., Jalal R., Toluinia B., Asoodeh A., Darvish J. (2013) Evaluation of relationship between HNF-1α and GLP-1R polymorphisms and type 2 diabetes in a population living in northeast of Iran. Journal of Cell and Molecular Research 5(2): 54-59.
9. Sadeghi B., Nassiri M., Masoudi-Nejad A., Tahmoorespour M., Dehghani H., Ahmadi H. (2012) Computational Analyses for Identification Novel MicroRNAs from Cattle and Sheep. Journal of Cell and Molecular Research 4(2): 62-67.
10. Raeisi S. (2013) Molecular docking approach of monoamine oxidase B inhibitors for identifying new potential drugs: Insights into drug-protein interaction discovery. Journal of Cell and Molecular Research 5(1): 24-34.

Research Article

PAX6 (+5a) Expression in Adipose Tissue-Derived Mesenchymal Stem Cells Induces Differentiation to Retinal Ganglion Cells

Habib Rezanejad[1], Farhang Haddad[1*], Zahra-Soheila Soheili[2]**, Maryam M. Matin[1,3], Shahram Samiei[4], and Sepideh Zununi Vahed[5]

1. *Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran*
2. *National Institute of Genetic Engineering and Biotechnology, Tehran, Iran*
3. *Cell and Molecular Biotechnology Research Group, Institute of Biotechnology, Ferdowsi University of Mashhad, Mashhad, Iran*
4. *Iranian Blood Transfusion Organization and Research Center, Tehran, Iran*
5. *School of Advanced Biomedical Sciences, Tabriz University of Medical Sciences, Tabriz, Iran*

Received 26 December 2013 Accepted 17 January 2014

Abstract

Glaucoma remains one of the major causes of blindness in today's world. The progressive field of stem cells proposes an exciting potential for discovering novel therapies. Here, we report the development of an easy and high throughput method for differentiation of retinal ganglion cells (RGCs) and bipolar cells from human adipose tissue-derived mesenchymal stem cells (hADSCs) using *PAX6* (+5a) gene expression, a master gene in development of the vertebrate visual system. HADSCs were isolated from fat tissues and confirmed by their cell surface markers and differentiation potential into adipocytes and osteocytes lineages. Then, the coding region of human *PAX6* (+5a) gene was cloned and lentiviral particles were produced. HADSCs differentiation was characterized by morphological characteristics, qRT-PCR and immunocytochemistry (ICC). The hADSCs were isolated successfully with high yield and purity (99%). After 30 hours post transduction by pLEX-*PAX6* - pur lentiviral vectors in fibronectin supplemented medium, cells gradually showed the characteristic morphology of neuronal cells. QRT- PCR and ICC confirmed deriving of mainly RGCs and marginally bipolar cells. The current investigation demonstrates the feasibility of differentiation of RGCs and bipolar cells from hADSCs using expression of *PAX6* (+5a) in the medium supplemented by fibronectin.

***Keywords*:** *PAX6* gene; adipose tissue-derived mesenchymal stem cells; retinal ganglion cell; transdifferentiation

Introduction

Glaucoma, a chronic retinal neurodegenerative disease, is the second cause of worldwide blindness in developed countries (Quigley and Broman, 2006). It has been estimated that 80 million people worldwide would have been affected by this disease by 2020. Glaucoma is characterized by the degeneration of axons in the optic nerve and apoptosis in retinal ganglion cells (RGCs) (Kuehn et al., 2005). RGCs are the first differentiating retinal cells in all vertebrates that are induced from retinal progenitor cells in embryonic development (Marquardt and Gruss, 2002). The inability of the central nervous system to regenerate new cellular components in response to damage leads to a limited capacity of structural and functional repair in the retina (Cao et al., 2002).

One of the strategies to restore vision in glaucoma patients is the functional replacement of RGCs using autologous or heterologous transplantation (Baker and Brown, 2009; MacLaren et al., 2006; Moshiri et al., 2004; Wallace, 2007; Wong et al., 2011). For the generation and transplantation of RGCs and their precursors, the potential of different sources of stem cells, including human embryonic stem cells (hESC), bone marrow-derived stem cells, umbilical cord-derived cells, induced pluripotent stem cells (iPS), adult human Müller stem cells and fetal stem cells, have been reported (Baker and Brown, 2009; Buchholz et al., 2013; Jayaram et al., 2011; John et al., 2013; Ramsden et al., 2013; Wallace, 2007).

Adipose tissue represents an abundant and accessible source of adult stem cells (Jurgens et al., 2008; Kokai et al., 2005). A growing body of experimental evidence, from *in vitro* and *in vivo* studies, demonstrates the multipotentiality of adipose-tissue derived stem cells (ADSCs) (Gimble et al., 2007).

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

While ADSCs are similar to bone marrow stem cells in differentiation and therapeutic potential, they are much easier and safer to obtain in large quantities which make them an ideal and reliable source for regenerative medicine (Rasmussen et al., 2012). The Paired box (PAX) genes are members of the family of tissue specific transcription factors. This family plays a critical role in the formation of tissues and organs during embryonic development and it has vital functions in certain tissues in adults (Hever et al., 2006; Pichaud and Desplan, 2002). *PAX6* along with other genes, such as SOX2 and OTX2, control each stage of eye development and it has been called the master gene for eye development (Hever et al., 2006). *PAX6* mutations in Drosophila, mouse, rat, and human demonstrate its requirement for the development of eye (Philips et al., 2005; Yasuda et al., 2002). *PAX6* (-5a) and *PAX6* (+5a) are two isoforms of the conserved *PAX6* gene that have different DNA binding specificities and functions (Azuma et al., 2005). It has been reported that *PAX6* (-5a) plays important roles in both embryogenesis and adult body homeostasis and *PAX6* (+5a) is the most critical isoform that promotes the neuronal differentiation of murine embryonic stem cells (Shimizu et al., 2009).

Based on these findings, it was considered that *PAX6* (+5a) transfection into hADSCs may induce retinal neurons, including RGCs and/or their precursors. In so doing, the expression of human *PAX6* (+5a) by lentiviral expression vectors was employed in hADSCs under a culture medium supplemented with fibronectin. This study offers an effective method for *in vitro* induction of retinal ganglion like cells that can be used in stem cell based therapy.

Materials and Methods

Adipose tissue sampling

Human adipose tissues were obtained from abdominal subcutaneous tissues of patients undergoing abdominoplasty procedures, in Tehran Medical University (N=6, age: 20-35 years). Before the surgical procedure, informed consents were obtained from the patients. About 150 ml of lipoaspirate was gathered in a sterile bottle, filled with 0.1 M phosphate-buffered saline (PBS) or DMEM(Dulbecco's modified Eagle's medium)-F12 (Sigma, Germany), in order to achieve enough number of cells.

Isolation and cell culture of hADSCs

HADSCs isolation was done according to previously published methods (Estes et al., 2010) with some modifications. Up to 2×10^7 adipose stromal stem cells with more than 98% purity were isolated from 150 ml of lipoaspirates; however, yields varied among patient's samples. Briefly, in order to remove the majority of erythrocytes and leukocytes, the lipoaspirate was washed five times with sterile PBS containing 120 µg/ml penicillin (Fluka, China) and 220 µg/ml streptomycin (Fluka, China). Then, 0.1% (wt/vol) collagenase type I (Invitrogen, USA) was used to digest the extracellular matrix. Enzyme activity was neutralized with DMEM supplemented fetal bovine serum (FBS) 10% (Gibco, Germany) and centrifuged at 1500 rpm for 10 min to obtain a high density pellet. The cell pellet was re-suspended and extensively washed with PBS. Finally, remaining cells were cultured in 25 cm^2 flasks (Nunc, Denmark). DMEM-F12 medium supplemented with 10% FBS, 5 ng/ml human epidermal growth factor (Roche, Germany), 1 ng/ml human fibroblastic growth factor (Roche, Germany), 100 U/ml penicillin and100 mg/L streptomycin was used as culturing and expansion medium. The flasks were then incubated at 37 °C with 5 % CO_2 in a humidified atmosphere. The medium was changed after 16 hours, and then twice a week. Adherent cells were harvested with 0.25% trypsin-0.02% EDTA, and re-plated at a dilution of 1:3 when the confluency was more than 80%.

Flowcytometric cell surface marker expression analysis

To characterize the phenotype of the cultured cells with flow cytometry, fluorescein isothiocyanate (FITC)-conjugated primary antibodies for human CD44, CD45, CD73, CD90 and CD105 (BD Biosciences, USA) were used. The hADSCs were trypsinized and washed three times with cooled PBS containing 2% FBS and sodium azide. Cells (5×10^4) were incubated with aforementioned antibodies. All antibodies were diluted 1:1000 and incubated with cells for 45 min at 4 °C. Then the cells were washed with PBS containing 2% FBS. After two washing steps, cells were re-suspended in 500 µl paraformaldehyde 2% containing 1% FBS for profile characterization and analyzing by fluorescence-activated cell sorting (FACS) system (Partec II, Germany).

Analysis of multipotent differentiation capacity

When over 80% confluency was reached, cells were incubated in the osteogenic and adipogenic differentiation media for four and three weeks, respectively. Osteogenic differentiation medium consisted of DMEM-F12, 20% FBS with

osteogenic supplement: 100 nM dexamethasone (Sigma-Aldrich, Germany), 50 µg/ml ascorbate-2-phosphate (Sigma-Aldrich, Germany) and 10 mM β-glycerolphosphate (Sigma-Aldrich, Germany). Adipogenic differentiation medium consisted of DMEM-F12 supplemented with 10% FBS, 100 nM dexamethasone (Sigma-Aldrich, Germany), 50 µg/ml indomethacin (Sigma-Aldrich, Germany) and 50 µg/ml ascorbate- 2 phosphate (Sigma-Aldrich, Germany). In general, the culture media were changed every 4 days. After induction, alkaline phosphatase assay (Sigma-Aldrich, Germany) and Oil Red O (Sigma-Aldrich, Germany) staining were performed to confirm the differentiation of hADSCs toward osteogenic and adipogenic lineages, respectively.

Construction of vectors

The coding sequence of human *PAX6* (+5a) gene was synthesized and cloned into pUC57 cloning vector. Vector was digested by *BamHI* and *XhoI* restriction enzymes and subcloned into the *BamHI/XhoI* site of pLEX-MCS-Pur lentiviral expression vector and was designated as pLEX-*PAX6* -Pur construct. The recombinant construct was confirmed by PCR ampilification, digestion and finally DNA sequencing.

Virus particles production

Lentiviral particles were produced by calcium-phosphate transient transfection using a vector expression system in HEK 293T cells. Briefly, HEK 293T cells were plated in 6-cm plates with 7×10^5 cells in 4 ml of DMEM-HG (high-glucose) supplemented with 10% FBS. Then pLEX-*PAX6* -Pur vector, as transfer vector (11 µg), envelope encoding plasmid (4 µg) and packaging lentivirus vector (7 µg) were added and transfection was carried out using $CaCl_2$. Fourteen hours after transfection, medium was replaced, and the supernatant was collected 24 and 48 hours post transfection. Finally, assembled lentiviral particles were filtered, purified and concentrated by PEG 6000 (Sigma, Germany).

Transduction of hADSCs

HADSCs were transduced by 8 hour exposure to the viral supernatant in the presence of 8 µg/ml polybrene at 37 °C and 5% CO_2 without FBS. 72 hours post transduction, selection medium containing DMEM-F12 with 20% FBS and 1 µg/ml puromycin was added to the transduced-hADSCs. Every 3 days the selective medium was changed with the same medium. Transduced-hADSCs from passages 2 post transduction were analyzed for eGFP expression, cell proliferation and cell death using ELISA kits (Roche, Germany) according to the manufacturer's instructions.

Real-time RT-PCR assay

Total RNA extraction and cDNA synthesis were performed using RNeasy kit (Qiagen, Germany), and Quantiscript® reverse transcriptase (Qiagen, Germany), respectively. Quantitative real-time PCR was performed with the Corbet Real-Time PCR system (Applied Biosystems, USA). Specific primers were used from the Quantitect primer assay (Qiagen, Germany) (Table 1). Data were normalized to the expression of *GAPDH*, a housekeeping gene, which has shown to have stable expression under different experimental conditions in similar studies. Each reaction contained 5 µl of Quantifast syber green master mix, 1 µl of forward and reverse mix primers (10 pM), 3 µl of RNase free water and 1 µl of cDNA. The reactions were conducted with initial enzyme activation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95°C for 10 seconds and anneal at 60 °C for 30s. Relative gene expression was calculated using Bio-Rad software (RelQuant UpDate- for relative quantification) according to the 2-ΔΔCt method based on the threshold cycle (Ct) values (Schmittgen and Livak, 2008). All experiments were performed in duplicate and their values were presented as mean±SD. Student's t-test was used to evaluate the statistical significance of the data; $p< 0.05$ was considered statistically significant.

Table. 1 primers list used for qPCR from quantitect primer assay (Qiagen, Germany)

	Official symbol	Amplicon length (bp)	Cat. No.
1	*PAX6*	113	QT00071169
2	FGF2	109	QT00047579
3	DVL3	69	QT00999810
4	SHH	136	QT00205625
5	SOX1	96	QT00215299

Immunocytochemistry

Immunocytochemical analysis was carried out for the detection of cell specific markers according to the Santa Cruz protocol with some modifications. Briefly, transduced hADSCs were cultured on FBS pre-coated glass cover slips in a 24-well microplate at a density of 6×104 cells per well and washed with PBS. Paraformaldehyde fixed cells were permeablized with chilled methanol (Merck, Germany) and then blocked in 1% BSA (Merck) in PBST (1% Triton X-100 in PBS) (Sigma) for 45 min at room temperature, followed by 1 h incubation in primary antibodies at room temperature (All antibodies were obtained from

Santa Cruz, USA). Antibodies for retinal progenitor and retinal ganglion and biopolar cell markers included the goat polyclonal anti-human *PAX6* , Thy1 and PKC. A negative secondary antibody-only control was also included. Nuclei were counter-stained with DAPI (1 mg/ml, Santa Cruz, USA) to assess the total number of cells in each field. Cover slips were then mounted onto slides using an anti-fading mounting medium (90% glycerol, 10% PBS and 10% (w/v) phenylene-diamine). Samples were observed under the Axiophot Zeiss fluorescence microscope (Germany) with a 460 nm filter for DAPI and a 520 nm filter for FITC-conjugated antibodies, and digital pictures were taken.

Results

C hADSCs

The hADSCs were isolated successfully from human adipose tissues. Approximately 5×10^6 hADSCs were obtained from one gram of adipose tissue. Cells were cultured in expansion medium and passaged every 3-4 days. Human ADSCs were large, spindle-shaped cells with fibroblastic features (Figure 1A, B). Thin cell body of cells contained a large and round nucleus (Figure 1C, D). In early passages, cells displayed clonogenic properties, the ability of a single cell to proliferate independently to form a colony (Figure 1E, F). This indicated the renewing capacity of isolated stem cells. Cells kept their morphological features, without major alteration, for a maximum of 11 passages.

Analysis of multipotent differentiation capacity

Osteogenic and adipogenic differentiation capacity of hADSCs were examined. Osteogenic differentiation was confirmed by mineralization of cells in osteogenic medium at week 4, which could be observed by alizarin red staining (Figure 1 G, H). Long spindle-shape morphology of the hADSCs changed into a polygonal shape 4 days after incubation in adipogenic induction medium. By day 9, small droplets of oil lipid appeared in some of the cells. After three weeks, most of the differentiated cells showed red lipid droplets throughout the cytoplasm, which was confirmed by oil red O staining (Figure 1 I, J).

Analysis of cell surface marker

To characterize cell surface markers of isolated cells, flow cytometry was performed. The flow cytometric analysis demonstrated that approximately 99% of hADSCs expressed the surface markers CD44, CD73 and CD105 (Figure 2A-C, G). The hADSCs lacked the expression of the hematopoietic markers CD34 and CD45 (Figure 2E, F). Results verified the mesenchymal origin of the hADSCs and the lack of hematopoietic markers. Each value represents the mean of two independent experiments in at least duplicate.

Successfully gene transduction of hADSCs by lentiviral vectors

Two lentiviral vectors pLEX-eGFP-pur and

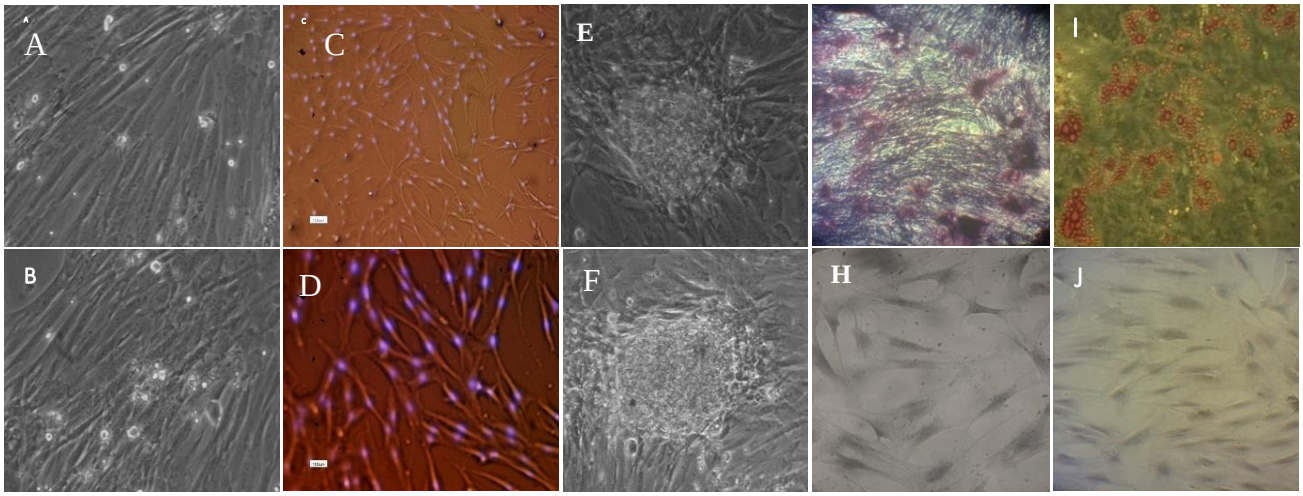

Figure 1. Characteristics of isolated hADSCs. Morphological features of cells (A-D); the hADSCs were typical fibroblast-like cells with fusiform shape from the 2nd passage and preserved their shape after expansion in vitro (A, B), Nuclei were stained with DAPI (blue), the cell body contained a large and round nucleus (C, D). Clonogenic capacity of hADSCs (E, F). Multipotential differentiation assays of hADSCs (G-J); differentiation potential of hADSCs towards osteogenic lineages was assessed through alkaline phosphatase activity assay (G, H), hADSCs differentiated towards the adipogenic lineage and formed lipid vesicles, which were stained using oil red-O (I, J). (C 100X – all other 200 X)

pLEX-*PAX6* -pur, were constructed and transfered into HEK293T and hADSCs. Transduction efficiency of hADSCs was examined by expression of GFP. Lentiviral vector, pLEX-eGFP-pur, was used to transduce proliferating hADSCs at a MOI of 100. One day after transduction, 90% and 75% of HEK293T and hADSCs expressed GFP, respectively. Although, the expression of GFP was observed only 24 hour post transduction in HEK293 cells, it took about 3 days to observe GFP in hADSC cells (Figure 3 A, B).

Cell morphology characteristics after transduction

Three days post transduction, cells showed the characteristic morphology of neuronal cells and little axon-like processes emerged gradually. Four days post transduction, they gradually extended axon-like processes that finally led to the formation of neural-network-like structures. Cells had multiple dendrites with a long axon and a fat cell body resembling reliable RGCs (Figure 3 C-F).

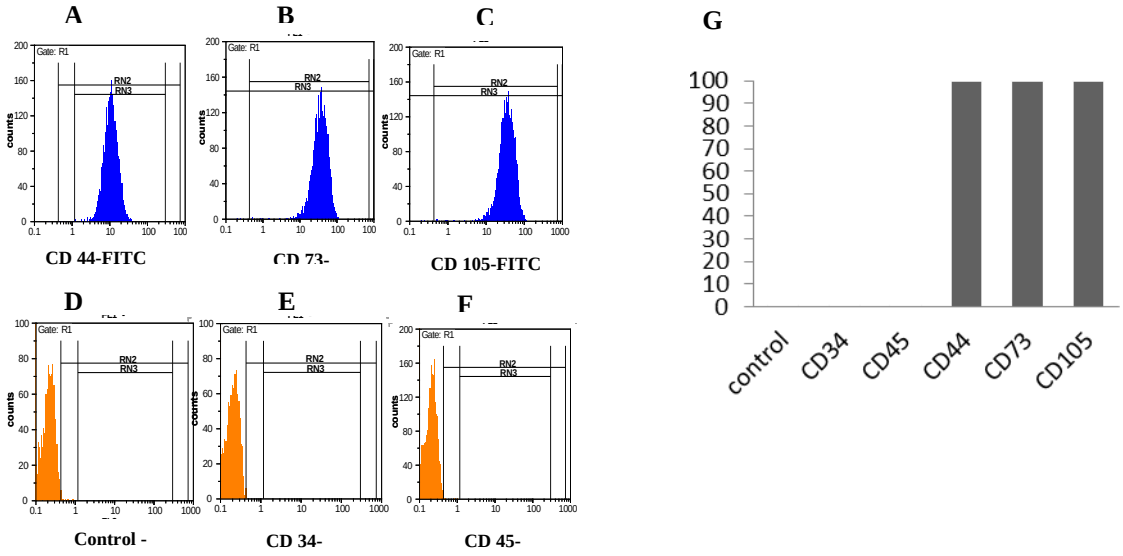

Figure 2. Flow cytometry analysis of hADSCs isolated from fat tissue and cultured *in vitro*. Immunophenotypic characterization of hADSCs (A-F), with cells positively expressing the antigens CD44 (A), CD73 (B) and CD105 (C), while negatively expressing the antigens CD34 (E) and CD45 (F), 99% of isolated hADSCs expressed the surface markers: CD44, CD73 and CD105 (G).

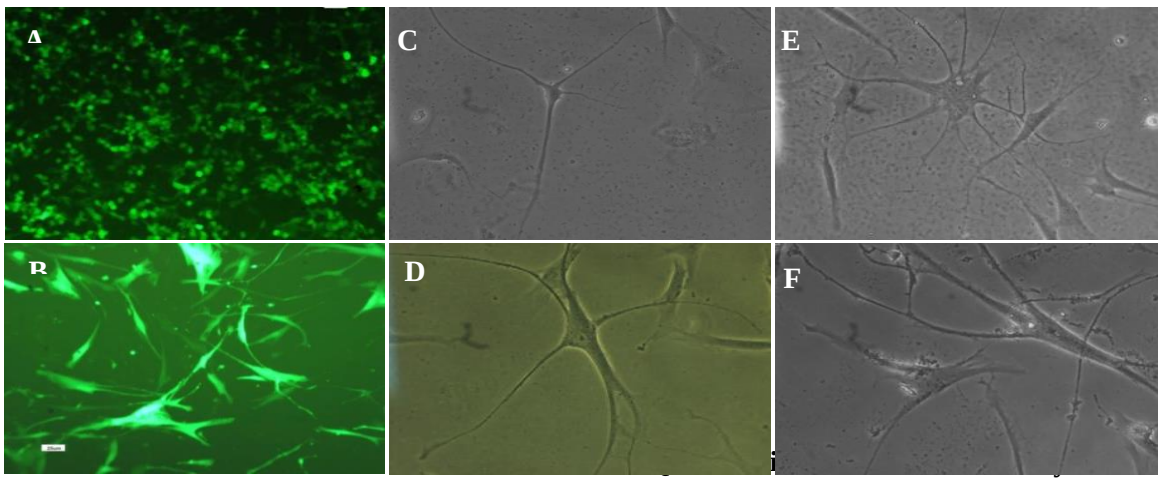

Figure 3. Transduction of hADSCs with lentiviral vectors. Efficient eGFP transduction of cells (A, B); HEK293T (A) and hADSCs (B) transduced with pLEX-eGFP-pur vector were expressing GFP. Cell morphology of transduced cell at day 4 under inverted microscopic view (C-F) (A 100X, all others 300X).

Real time RT-PCR analysis

To test the expression of retinal neuron and RGC-associated mRNAs, including *SOX1*, *PAX6*, *Thy1* and *PKC*, qRT-PCR was performed. According to the qRT-PCR data, *PAX6* and *SOX1* expression levels increased substantially after transduction compared to cells transduced by empty vector or without transduction. Thy1, a marker of RGCs, plays an important role in the formation of the visual system. Expression of *FGF2* and *sonic hedgehog* (*SHH*) as molecules involved in different signaling pathways in differentiation of retinal neural cells was also examined. Results showed the increased expression of *FGF2* and *SHH* (Figure 4). At the mRNA level, expression of *PKC*, a bipolar cell marker, was also increased. Taken together, these results indicated that *PAX6* (+5a) induction of hADSCs can cause differentiation into mostly RGCs and marginally bipolar cells.

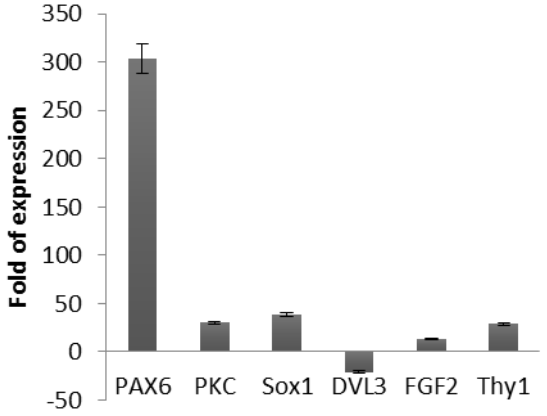

Figure 4: Relative retinal gene expression in *PAX6* (5a)-transduced hADSCs after 6 days in comparison to the cells transduced by pLEX-MCS-pur (empty vector). The results represent the mean of 3 independent experiments in at least triplicate.

ICC confirmed the expression of some key markers of RGCs.

To determine whether PAX6 (+5a) transcription factor and fibronectin were able to induce hADSCs into retinal cells, we examined the expression of *PAX6*, *Thy1* and PKC markers in *PAX6* (+5a)-transduced cells. Using ICC method, cells were positive for *PAX* (64± 1.3%), Thy1 (32± 1.1%) and PKC (29± 1.4%) (Figure 5 A-F). Cells transfected with empty vector did not show any of these markers.

Discussion

Glaucoma is the most common cause of irreversible blindness in the world. Despite the advances in the currently available treatments, many patients experience significant visual loss due to degeneration of RGC (Kerrigan–Baumrind et al., 2000; Kuehn et al., 2005; Quigley and Broman, 2006). Right now, there is no therapeutic strategy for functional recovery of these cells. Cell therapy offers an alternative treatment for restoring the damaged cells in neurodegenerative diseases (Buchholz et al., 2013; Cao et al., 2002; Haddad-Mashadrizeh et al., 2013; Huang et al., 2013; Huang et al., 2011; John et al., 2013). It is acknowledged that damage to the neural retina during glaucoma is restricted to the degeneration of RGCs (Kerrigan–Baumrind et al., 2000); therefore, replacement of these cells might be possible and, if so, might restore the optic nerve.

Several groups have focused on the differentiation of stem cells from different sources with various methods (Fraichard et al., 1995;

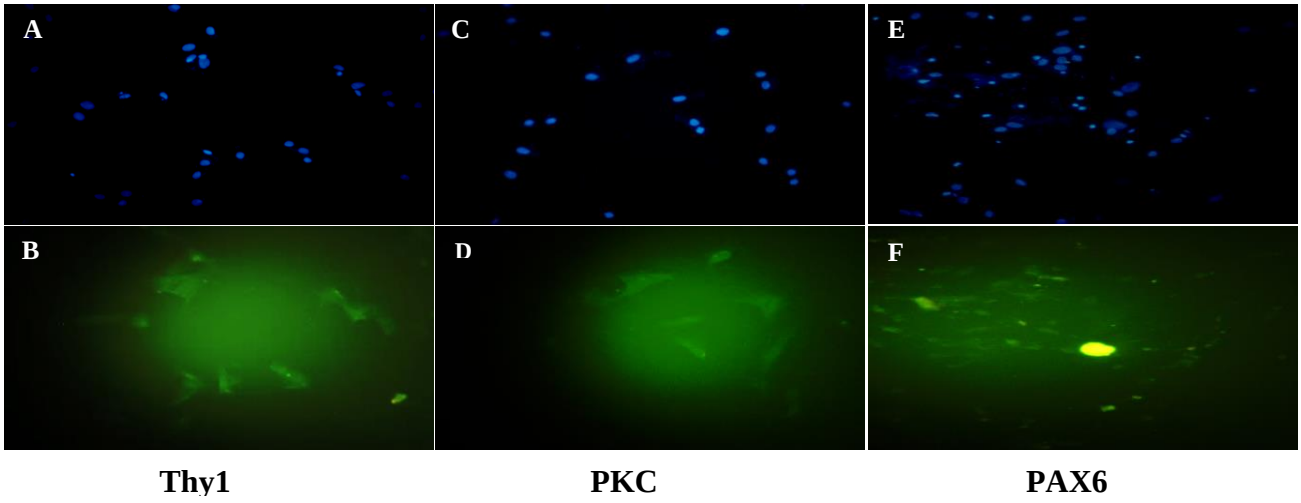

Figure 5. Fluorescence microscopy of Thy1, PKC and PAX6 in Pax6 (+5a)- transduced cells after 6 days. These Pax6 (+5a)-transduced cells were analyzed by in situ immunostaining with antibodies raised against different RGC markers. A,B) ICC for Thy1 C,D) Antibody against PKC E,F) antibody against Pax6. Each value represents the mean of 3 independent experiments in at least triplicate.

Jagatha et al., 2009; Jin et al., 2009; Osakada et al., 2009; Singhal et al., 2012; Wong et al., 2011). Most of these sources have limitations for human RGC replacement in clinics (Jayaram et al., 2011). Consequently, identifying alternative sources of cells that replace these cells in the glaucomatous eye without ethical and practical restrictions is necessary. With this target in mind, this study aimed to address some of these limitations.

This study established an effective and easy way to obtain high-yield hADSCs with high purity, 99% positive staining rate observed from results of multiple cell surface markers, using collagenase digestion and adherence screening. No significant difference was seen among different passages in the phenotypes of hADSCs, indicating that the cells can be stably amplified *in vitro* for several passages. High proliferation (data not shown) and differentiation (Figure 1G-J) capacity of isolated hADSCs is consistent with stem cell characteristics. It is reported that fibronectin may be important for differentiating ESCs into retinal neuron precursors, including RGC-like cells (Kayama et al., 2010). Based on these findings, culture medium was supplemented with fibronectin for neural cell induction. Results showed that *PAX6* (+5a) expression and fibronectin supplemented medium are sufficient to induce the differentiation of retinal precursor cells from hADSCs.

Inverted microscopic examination disclosed the appearance of generally long cells that exhibited multidendrites and one axon per cell, suggesting their neuronal differentiation (Figure 3C-F). Several genes expressed in differentiating RGCs such as *SOX1*, *PAX6*, and *Thy1* were tested by qPCR. The results revealed that upon differentiation, the cells had up-regulated expression of early neural markers, SOX1 and *PAX6*. SOX1 is one of the earliest transcription factors that is expressed in cells committed to the neural fate (Pevny et al., 1998). *PAX6* is a neural/retinal progenitor marker and acts as a master switch for activation of RGC regulator, thereby supposed to initiate the RGC differentiation cascade (Jagatha et al., 2009). Thy1, a surface glycoprotein, is uniquely expressed in RGCs in retina (Huang et al., 2006).

Different signaling molecules such as *FGF2* and *SHH* have been shown to be involved in differentiation of RGCs. It has been shown that FGF2 is a potent stimulator of axon growth during RGC development (Sapieha et al., 2003). Sonic hedgehog (Shh) has been shown to play an important role in the development of the retina in a number of different model organisms (Spence et al., 2004). Recent studies have demonstrated that the signaling molecule Shh secreted by differentiated RGCs is required to promote the progression of ganglion cell differentiation. Shh plays dual roles to orchestrate the progression of retinal neurogenic wave (Zhang and Yang, 2001) and also plays a major role in RGC axon projection inside the retina (Kolpak et al., 2005). FGF2 and SHh are known to activate *PAX6* (Jagatha et al., 2009). Increased levels of FGF2 and SHh and *PAX6* in this study were in line with these findings.

Since RGCs collect the messages from bipolar cells and represent the ultimate signals to the vision center in the brain, mRNA expression of PKC (protein kinase C), a bipolar cell marker, was also examined. Expression of *SOX1*, *PAX6* , *FGF2*, *Thy1*, *SHH* along with ICC results of *PAX6* , Thy1 and PKC confirmed that the differentiated cells belonged to RGCs and bipolar cells (Figure 4 and 5). Major disadvantages associated with the use of different sources of stem cells are their ethical concerns, shortage of donor cells, limited availability, inflammation, immunoreaction as well as their safety issues regarding teratoma formation; therefore, their potential in cell therapy may be problematic (Wong et al., 2011). Human ADSCs can overcome some of these problems.

Aside from developing a reliable source of retinal cells for transplantation, there are several additional obstacles that have to be considered. For instance, limitation of integration of graft cells into host tissue (Wong et al., 2011), ongoing disease in the host environment that may present a problem for cell transplantation (Wallace, 2007) and tumorigenicity, specially, when cell cultivation period has prolonged (Wong et al., 2011). Moreover, after the transplantation of RGCs, it would need the additional challenge of regrowth of axons through the optic nerve to targets in the brain (Wallace, 2007).

Taken together, for successful retinal regeneration, improved methods for purifying donor retinal cells, optimizing host conditions, as well as using animal models of human diseases, to determine the efficacy and safety of treatments, will be crucial. Furthermore, in order to optimize the best minimal cocktail requested to achieve more authentic-differentiated neurons, more growth factors, cytokines, mRNA, microRNA and small molecules deserve to be investigated.

Future studies to determine the markers of differentiated cells and also time course expression of aforementioned genes are under investigation. Using current method, bipolar cells and RGCs beside different retinal cells including photoreceptors (our previous work, in press) were successfully differentiated from hADSCs. For an efficient differentiation of hADSCs to disease-relevant cell types, novel strategies need to be developed. The current investigation demonstrates the feasibility of the differentiation of RGCs and bipolar cells from hADSCs using expression of *PAX6* (+5a) in the medium supplemented by fibronectin that can be used in stem cell therapy.

Acknowledgement

We would like to thank Ferdowsi University of Mashhad, Mashhad, Iran for their Grant # 30368 to perform this work.

References

1- Azuma N., Tadokoro K., Asaka A., Yamada M., Yamaguchi Y., Handa H., Matsushima S., Watanabe T., Kohsaka S., Kida Y., Shiraishi T., Ogura T., Shimamura K. and Nakafuku M. (2005) The *PAX6* isoform bearing an alternative spliced exon promotes the development of the neural retinal structure. Human molecular genetics 14:735-745.

2- Baker P. S. and Brown G. C. (2009) Stem-cell therapy in retinal disease. Curr Opin Ophthalmol 20:175-181.

3- Buchholz D. E., Pennington B. O., Croze R. H., Hinman C. R., Coffey P. J. and Clegg D. O. (2013) Rapid and efficient directed differentiation of human pluripotent stem cells into retinal pigmented epithelium. Stem Cells Translational Medicine 2:384-393.

4- Cao Q., Benton R. L. and Whittemore S. R. (2002) Stem cell repair of central nervous system injury. Journal of neuroscience research 68:501-510.

5- Estes B. T., Diekman B. O., Gimble J. M. and Guilak F. (2010) Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc 5:1294-1311.

6- Fraichard A., Chassande O., Bilbaut G., Dehay C., Savatier P. and Samarut J. (1995) *In vitro* differentiation of embryonic stem cells into glial cells and functional neurons. J Cell Sci 108 (Pt 10):3181-3188.

7- Gimble J. M., Katz A. J. and Bunnell B. A. (2007) Adipose-derived stem cells for regenerative medicine. Circulation research 100:1249-1260.

8- Haddad-Mashadrizeh A., Bahrami A. R., Matin M. M., Edalatmanesh M. A., Zomorodipour A., Gardaneh M., Farshchian M. and Momeni-Moghaddam M. (2013) Human adipose-derived mesenchymal stem cells can survive and integrate into the adult rat eye following xenotransplantation. Xenotransplantation 20:165–176.

9- Hever A., Williamson K. and Van Heyningen V. (2006) Developmental malformations of the eye: the role of *PAX6* , SOX2 and OTX2. Clinical genetics 69:459-470.

10- Huang L., Liang J., Geng Y., Tsang W. M., Yao X., Jhanji V., Zhang M., Cheung H. S., Pang C. P. and Yam G. H. (2013) Directing adult human periodontal ligament-derived stem cells to retinal fate. Investigative Ophthalmology and Visual Science 54:3965-3974.

11- Huang W., Fileta J., Guo Y. and Grosskreutz C. L. (2006) Downregulation of Thy1 in retinal ganglion cells in experimental glaucoma. Current eye research 31:265-271.

12- Huang Y., Enzmann V. and Ildstad S. T. (2011) Stem cell-based therapeutic applications in retinal degenerative diseases. Stem Cell Rev 7:434-445.

13- Jagatha B., Divya M. S., Sanalkumar R., Indulekha C. L., Vidyanand S., Divya T. S., Das A. V. and James J. (2009) *In vitro* differentiation of retinal ganglion-like cells from embryonic stem cell derived neural progenitors. Biochemical and biophysical research communications 380:230-235.

14- Jayaram H., Becker S. and Limb G. A. (2011) Stem Cell Based Therapies for Glaucoma.

15- Jin Z. B., Okamoto S., Mandai M. and Takahashi M. (2009) Induced pluripotent stem cells for retinal degenerative diseases: a new perspective on the challenges. J Genet 88:417-424.

16- John S., Natarajan S., Parikumar P., Shanmugam P. M., Senthilkumar R., Green D. W. and Abraham S. J. (2013) Choice of Cell Source in Cell-Based Therapies for Retinal Damage due to Age-Related Macular Degeneration: A Review. J Ophthalmol 2013:465169.

17- Jurgens W. J., Oedayrajsingh-Varma M. J., Helder M. N., Zandiehdoulabi B., Schouten T. E., Kuik D. J., Ritt M. J. and van Milligen F. J. (2008) Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. Cell and tissue research 332:415-426.

18- Kayama M., Kurokawa M. S., Ueda Y., Ueno H., Kumagai Y., Chiba S., Takada E., Ueno S., Tadokoro M. and Suzuki N. (2010) Transfection with *PAX6* gene of mouse embryonic stem cells and subsequent cell cloning induced retinal neuron

progenitors, including retinal ganglion cell-like cells, *in vitro*. Ophthalmic research 43:79-91.

19- Kerrigan–Baumrind L. A., Quigley H. A., Pease M. E., Kerrigan D. F. and Mitchell R. S. (2000) Number of ganglion cells in glaucoma eyes compared with threshold visual field tests in the same persons. Investigative Ophthalmology & Visual Science 41:741-748.

20- Kokai L. E., Rubin J. P. and Marra K. G. (2005) The potential of adipose-derived adult stem cells as a source of neuronal progenitor cells. Plast Reconstr Surg 116:1453-1460.

21- Kolpak A., Zhang J. and Bao Z. Z. (2005) Sonic hedgehog has a dual effect on the growth of retinal ganglion axons depending on its concentration. The Journal of neuroscience 25:3432-3441.

22- Kuehn M. H., Fingert J. H. and Kwon Y. H. (2005) Retinal ganglion cell death in glaucoma: mechanisms and neuroprotective strategies. development 1:3.

23- MacLaren R. E., Pearson R. A., MacNeil A., Douglas R. H., Salt T. E., Akimoto M., Swaroop A., Sowden J. C. and Ali R. R. (2006) Retinal repair by transplantation of photoreceptor precursors. Nature 444:203-207.

24- Marquardt T. and Gruss P. (2002) Generating neuronal diversity in the retina: one for nearly all. Trends in neurosciences 25:32-38.

25- Moshiri A., Close J. and Reh T. A. (2004) Retinal stem cells and regeneration. Int J Dev Biol 48:1003-1014.

26- Osakada F., Jin Z. B., Hirami Y., Ikeda H., Danjyo T., Watanabe K., Sasai Y. and Takahashi M. (2009) *In vitro* differentiation of retinal cells from human pluripotent stem cells by small-molecule induction. Journal of cell science 122:3169-3179.

27- Pevny L. H., Sockanathan S., Placzek M. and Lovell-Badge R. (1998) A role for SOX1 in neural determination. Development 125:1967-1978.

28- Philips G. T., Stair C. N., Young Lee H., Wroblewski E., Berberoglu M. A., Brown N. L. and Mastick G. S. (2005) Precocious retinal neurons: *PAX6* controls timing of differentiation and determination of cell type. Developmental biology 279:308-321.

29- Pichaud F. and Desplan C. (2002) Pax genes and eye organogenesis. Curr Opin Genet Dev 12:430-434.

30- Quigley H. A. and Broman A. T. (2006) The number of people with glaucoma worldwide in 2010 and 2020. British Journal of Ophthalmology 90:262-267.

31- Ramsden C. M., Powner M. B., Carr A. J., Smart M. J., da Cruz L. and Coffey P. J. (2013) Stem cells in retinal regeneration: past, present and future. Development 140:2576-2585.

32- Rasmussen J. G., Frobert O., Holst-Hansen C., Kastrup J., Baandrup U., Zachar V., Fink T. and Simonsen U. (2012) Comparisson of human adipose- derived stem cells and bone marrow-derived stem cells in a myocardial infarction model. Cell Transplant.

33- Sapieha P. S., Peltier M., Rendahl K. G., Manning W. C. and Di Polo A. (2003) Fibroblast growth factor-2 gene delivery stimulates axon growth by adult retinal ganglion cells after acute optic nerve injury. Molecular and Cellular Neuroscience 24:656-672.

34- Schmittgen T. D. and Livak K. J. (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101-1108.

35- Shimizu N., Watanabe H., Kubota J., Wu J., Saito R., Yokoi T., Era T., Iwatsubo T., Watanabe T., Nishina S., Azuma N., Katada T. and Nishina H. (2009) *PAX6* -5a promotes neuronal differentiation of murine embryonic stem cells. Biol Pharm Bull 32:999-1003.

36- Singhal S., Bhatia B., Jayaram H., Becker S., Jones M. F., Cottrill P. B., Khaw P. T., Salt T. E. and Limb G. A. (2012) Human Müller Glia with Stem Cell Characteristics Differentiate into Retinal Ganglion Cell (RGC) Precursors *In vitro* and Partially Restore RGC Function *In vivo* Following Transplantation. Stem cells translational medicine 1:188-199.

37- Spence J. R., Madhavan M., Ewing J. D., Jones D. K., Lehman B. M. and Del Rio-Tsonis K. (2004) The hedgehog pathway is a modulator of retina regeneration. Development 131:4607-4621.

38- Wallace V. A. (2007) Stem cells: a source for neuron repair in retinal disease. Canadian Journal of Ophthalmology/Journal Canadien d'Ophtalmologie 42:442-446.

39- Wong I. Y. H., Poon M. W., Pang R. T. W., Lian Q. and Wong D. (2011) Promises of stem cell therapy for retinal degenerative diseases. Graefe's Archive for Clinical and Experimental Ophthalmology 249:1439-1448.

40- Yasuda T., Kajimoto Y., Fujitani Y., Watada H., Yamamoto S., Watarai T., Umayahara Y., Matsuhisa M., Gorogawa S., Kuwayama Y., Tano Y., Yamasaki Y. and Hori M. (2002) *PAX6* mutation as a genetic factor common to aniridia and glucose intolerance. Diabetes 51:224-230.

41- Zhang X. M. and Yang X.-J. (2001) Regulation of retinal ganglion cell production by Sonic hedgehog. Development 128:943-957.

Research Article

Investigations on chromosome variation in *Achillea tenuifolia* Lam and *A. bieberestinii* Afan (Asteraceae) from Hamedan and Kermanshah in the West of Iran

Abdolkarim Chehregani-Rad*, Fariba Mohsenzadeh, Hajar Salehi

Laboratory of Plant Cell Biology, Department of Biology, Bu-Ali Sina University, Hamedan, Iran

Received 27 Nov 2013 Accepted 14 Jan 2014

Abstract

Chromosome counting was performed in nine populations of *Achillea tenuifolia* Lam and eight populations of *A. bieberestinii* Afan (Asteraceae) collected from Hamedan and Kermanshah provinces in the west of Iran. Chromosome numbers in both species varied from 2n=2x=18 to 2n=4x=36. Some populations of both species showed (2n=4x=36) chromosome number that is the first report as polyploidy levels. Aneuploidy is also the first report for both species. Diploid and tetraploid individuals were observed in some populations at the same locality. B-chromosomes were observed in some populations of both species. The results indicated that polyploidy is a common future in this species similar to other Asteraceae plants.

Keywords: *Achillea bieberestinii*, *A. tenuifolia*, Chromosome number, Polyploidy

Introduction

The Compositae (Asteraceae) is the largest and most successful flowering plant family in the world with about 1700 genfera and 25,000 species. They grow everywhere but they prefer open areas and are common garden plants (i.e., sunflowers, daisies, artichokes, thistles, lettuce) (Funk et al., 2009). Yarrow (Achillea L.) is one of the youngest evolutionary genera of the Asteraceae family, which is present throughout the world (Goli et al., 2008; Rahimmalek et al., 2009).

The genus Achillea L. has approximately 130 perennial herb species (Saukel et al., 2004; Guo et al., 2004, 2005). Most of the its species are distributed in Eurasia, some in North Africa, and a few can be found in North America and in the Southern Hemisphere (Post 1933; Bremer and Humphries 1993; Zheng-Yi and Raven 1994). Bumadaran is a popular name for several species of Achillea in Persian language. The majority of Achillea species are of medicinal values having therapeutic applications. They have been used as anti-inflammatory (Benedek and Kopp 2007), anti-spasmodic (Karamenderes and Apaydin 2003), diaphoretic, diuretic, emmenagogic agents and for treatment of hemorrhage, pneumonia, rheumatic pain and wounds since antiquity (Zargari 1996).

The most common basic chromosome number in the Anthemideae is x=9, although x=8 and x=10 have also been reported by some researchers (Carr et al., 1999; Vallès et al., 2005; Chehregani and Hajisadeghian 2009). The basic chromosome number in Achillea is x=9, with polyploidy (4x, 6x, 8x) occurring frequently that most of the species being diploid. The genus exhibits wide ecological ranging from deserts to aqueous habitats and from level of sea to the high mountains (Lawrence 1947; Contandriopoulos and Martin 1967; Liove 1972, 1973; Oswiecimska 1974; Halliday and Beadle 1980; Tutin et al., 1980; Androschchuk and Kostinenko 1981; Danihelka and Rotreklová 2001, 2002; Constantinidis and Kalpoutzakis 2005; Magulaev 1982; D¸abrowska 1989, 1992; Maffei et al. 1993; Ehrendorefer and Guo 2006; Chehregani et al., 2013). The genus is also widespread in different regions of the Iran, with 19 species (Podelech 1986), out of which 7 species are endemic to Iran (Mozaffarian 2005, 2007).

Polyploidy is currently considered as a prominent force in plant evolution and represents the most common mode of sympatric speciation in plants (Wendel and Doyle 2005 and references cited there in; Chehregani et al., 2010).

∗Corresponding author E-mail: chehregani@basu.ac.ir

Table 1- The populations of *Achillea tenuifolia* and *A. bieberestinii* that were subjected for karyological studies

Sr.#	Name of species	Locations	Geographical characters	Altitude (m)	Collector	Chromosome number	Date of collection
1	*Achillea tenuifolia*	Hamedan, Razan, Boghati mountain	N: 35^0 32.8' 58." E:48^0 43.1' 63"	2385	Salehi	*2n=18*	89.4.19
2	*A. tenuifoila*	Hamedan, Malayer to Arak road, Protected área of Lashkardar, Border Road	N: 34^0 08' 28.5" E: 48^0 13' 03.2"	1852	Salehi	*2n=36*	89.4.23
3	*A. tenuifoia*	Hamedan to Kermanshah, Near to Asadabad city	N: 34^0 49' 23.3" E: 48^0 11' 04.7"	2315	Salehi	*2n=18,36*	89.4.20
4	*A. tenuifolia*	Hamedan, 40 km from Asadabad to Kermanshah, Viilage of Tajabad, after Almagholagh mountain	N: 34^0 53' 15.8" E: 48^0 11' 66.6"	2012	Salehi	*2n=18,36*	89.420
5	*A. tenuifoila*	Hamedan, Kabudarahang, Soubashi mountans	N: 35^0 11' 61.5" E: 48^0 16' 24.7"	2295	Salehi	*2n=18,36*	89.4.16
6	*A. tenuifoila*	Hamedan, Road of Malayer to Arak, village of Marvil , Malvak mountain	N:34^0 02,2' 77.9" E: 48^0 50' 53.1"	2249	Salehi	*2n=16,36*	89.4.18
7	*A. tenuifolia*	Hamedan, Kabudarahang, Gholi abad mountain	N: 35^0 15' 15.1" E: 48^0 50' 30.1"	2304	Salehi	*2n=18,36*	89.4.22
8	*A. tenuifoila*	Hamedan, road of Malayer to Arak, village of Marvel, slopes of Malvak mountain	N: 34^0 02' 97.6" E: 48^0 50' 18	2148	Salehi	*2n=18,36*	89.43.18
9	*A. tenuifoila*	Hamedan, road of Malayer to Arak, Ahangaran Lead and Zink mine area	N: 34^0 47 ' 29.2" E: 48^0 29' 19"	2003	Salehi	*2n=18,36*	89.4.18
10	*Achillea bieberestinii*	Hamedan to Kermanshah road, 40 km after Asadabad, village of Tajabad , after Almagholagh mountain, beside of raceway	N: 340 53' 15" E: 480 11' 65"	2012	Salehi	2n=18	89.4.20
11	*A. bieberestinii*	Hamedan city, Ganjnameh area	N:340 43' 90.3" E: 480 25' 88.7"	2356	Salehi	2n=18	89.4. 22
12	*A. biebrestinii*	Hamedan, Ganjnameh area, right sides of waterfall	N: 340 45' 82.0" E: 480 26' 23.9. "	2248	Salehi	2n=18,36	89.4.21
13	*A. bieberestinii*	Hamedan, Ganjnameh, 300m higher than waterfall, beside of Kiwarestan campus	N: 340 45' 93.9" E: 480 25' 91.1"	2367	Salehi	2n=18,36	89.4.21
14	*A. bieberestinii*	Hamedan, road of Ganjnameh ,the border of garden	N: 340 45' 52.6." E:480 26' 34.3"	2100	Salehi	2n=18,36	89.4.21
15	*A. bieberestinii*	Hamedan, village of Heiydarh, beside of road	N: 340 11' 15.1" E: 480 20' 15"	1890	Salehi	2n=18,36	89.4.22
16	*A. bieberestinii*	Hamedan, village of Moradbayg	N: 340 44' 89.4" E: 480 30' 17"	2059	Salehi	2n=18,36	89.4.21
17	*A. bieberestinii*	Hamedan, Nahavand city, Gian forest, right side of valley	N: 340 08' 03.5" E: 480 13' 05.2"	1730	Salehi	2n=18,36	89.4.18

Polyploidy and hybridization have been essential for massive secondary evolutionary radiation in Achillea as in many other angiosperm clades (Leitch and Bennett 1997; Wendel 2000; Soltis et al., 2003). Therefore polyploidy has an important role in the evolution and speciation in higher plants, which are estimated from 35 to 80% of all species (Stebbins 1971; Soltis and Soltis 2000). The native flora of Iran comprises about 8000 angiosperm species. Chromosome counts on Iranian material have so far been carried out for about 1500 species, but in many cases only a single chromosome count has been studied (Ghaffari and Kelich 2006).

An aneuploid is an unbalanced polyploidy in cells or individuals having chromosome numbers different than basic number (Rieger et al., 1968). In aneuploids changing of chromosome numbers are occurred and it involves the addition or subtraction of chromosomes or the gain or loss of genetic material by means of a centromere without changing the total number of chromosome (Moore 1976). According to the original definition, aneuploidy refers to organisms having one or two or a few chromosomes more or less than the basic chromosome number in the species (Dyer et al., 1970).

Nevertheless, Achillea species are poorly known in terms of karyology and ploidy level; and there are few chromosome counts that were reported different chromosome number for a species. Our aims in this paper was to contribute to the general knowledge of chromosome number in Achillea tenuifolia and A. bieberestinii, to provide more information about their chromosome number variation. Therefore, the main objective of the study was to determine chromosome number in the different populations of Achillea tenuifolia and A. bieberestinii growing in West provinces of Iran.

Materials and methods

Plant materials- Plant samples of Achillea tenuifolia and A. bieberestinii plants were collected from the populations growing in natural areas in the West of Iran, Hamedan and Kermanshah provinces. Specimens were prepared and dried in a local herbarium at Bu-Ali Sina University and were

determined using taxonomical keys and related references (Mozafarian 2005, 2007; Podlech 1986). Determination of the collected plant materials was also confirmed by the scientists at Rangeland Research Center of Hamedan.

Voucher specimens of the all studied materials were deposited in the Herbarium of Department of Biology, Faculty of Science, Bu-Ali Sina University, Iran (BHU). The locations, collectors and dates are shown in Table 1. Seeds were collected from at least ten plants in each population.

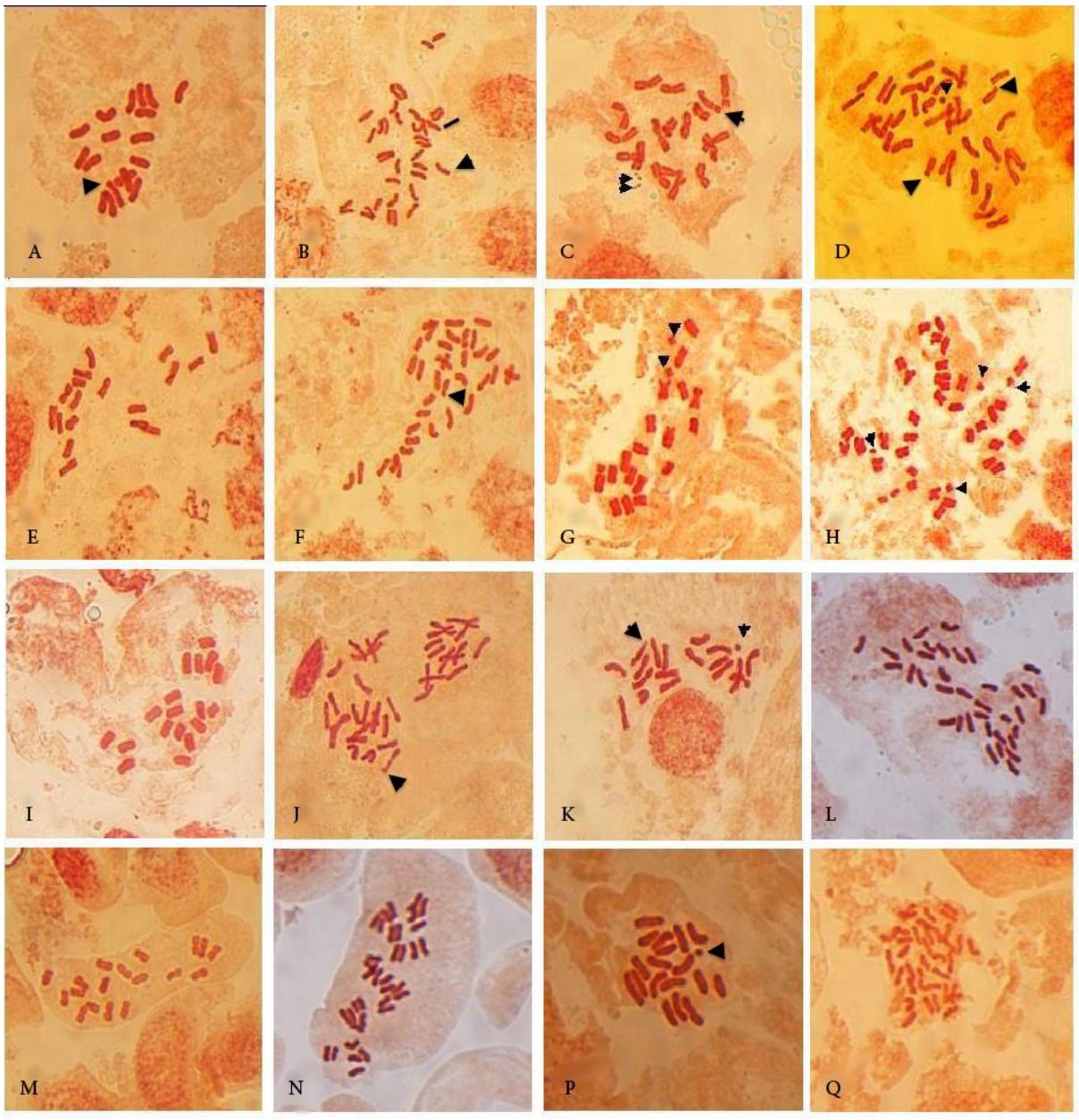

Fig. 1- Light micrographs of mitotic metaphase chromosomes prepared from different populations of Achillea tenuifolia. A, Population No. 1 with chromosome number 2n=2x=18; B, Population No. 2 with chromosome number 2n=4x=36. C-P, The populations 3-9 with two different chromosome numbers 2n=2x=18 and 2n=4x=36. Arrow indicated B-chromosomes or satelites chromosomes in the figures. Bar in all figures= 5µm.

Karyological studies: Chromosome counts were made on somatic metaphases using standard squash techniques. Seeds collected from wild samples were used in the present study. Root-tip meristems were obtained by germinating seeds on the wet filter paper in Petri dishes at approximately 20°C. Samples were pretreated with 0.05% colchicine for 2.5 h at room temperature. The materials were fixed in 3:1 v/v absolute ethanol: glacial acetic acid for a minimum of 24 h at 4°C.

Meristems were hydrolysed in 1 M hydrogen chloride (HCl) for 30 min at room temperature. They were then stained in 2% acetic orcein for a minimum of 3 h at 4°C (Chehregani et al. 2012). Squashes were made in 45% acetic acid. Photographs were taken through a Zeiss Axiostra microscope (Germany) with a Canon G11 (Japan) digital camera.

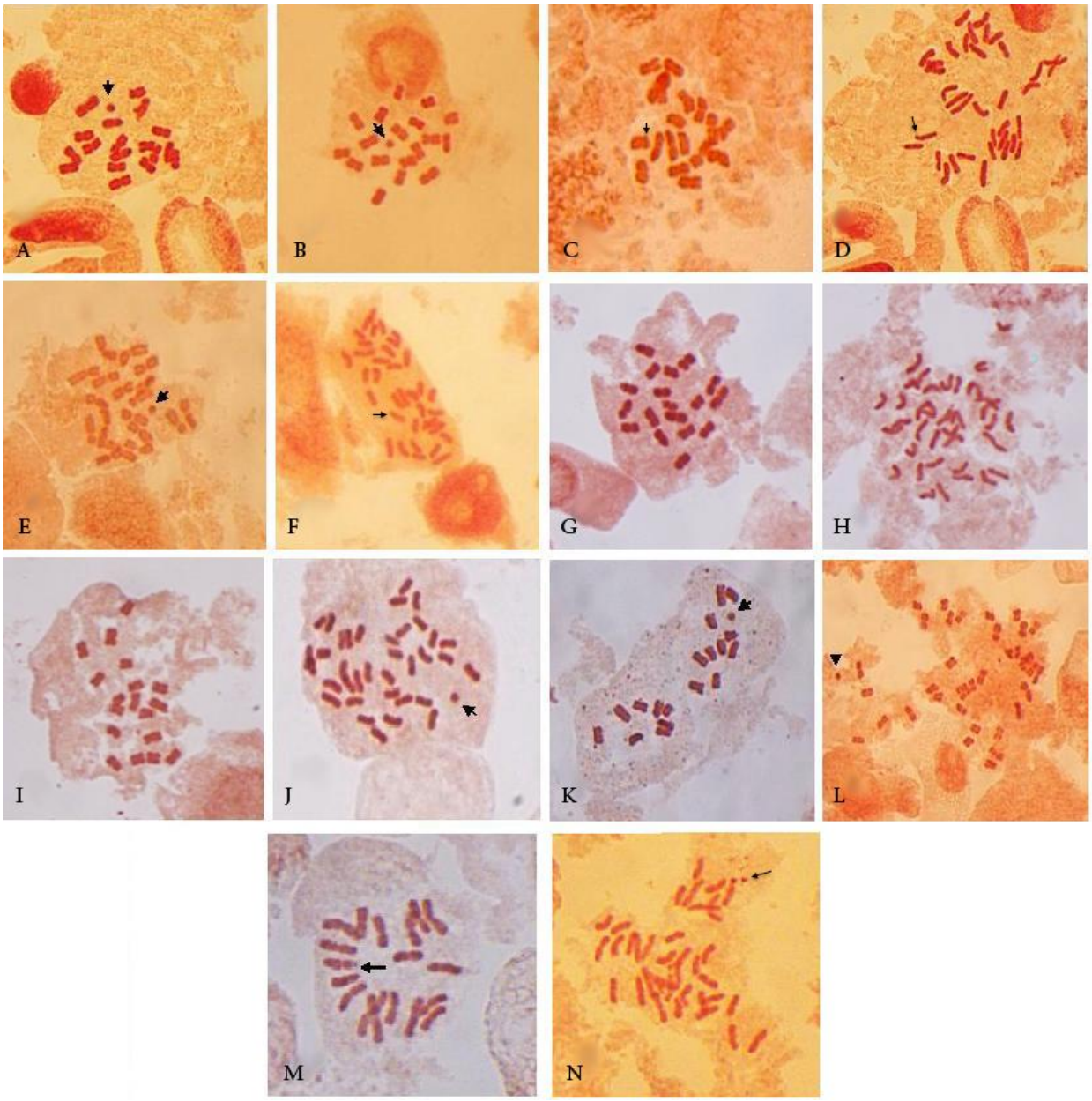

Fig. 2- Light micrographs of mitotic metaphase chromosomes prepared from different populations of Achillea biebersteinii. A, Population No. 1 with chromosome number 2n=2x=18; B, Population No. 2 with chromosome number 2n=2x=18. C-N, The populations 3-8 with two different chromosome numbers 2n=2x=18 and 2n=4x=36. Arrow indicated B-chromosomes or satelites chromosomes in the figures. Bar in all figures= 5µm.

Table 2-Somatic chromosome number in the 9 populations of *Achillea tenuifolia* and as detailed form.

Pop No.	Chromosome number / Name of species	18	36	17	16	15	19	Number of B chromosome	Number of Satellite
1	***A. tenuifolia***	18(8/10) 80%	-	17(1/10) 10%	-	15(1/10) 10%	-	-	-
2	***A. tenuifoila***	-	36(10/10) 100%	-	-	-	-	-	-
3	***Achillea tenuifoia***	18(37/50) 74%	36(13/50) 26%	-	-	-	-	(3/50)=6% 0-1B	(5/50)=10% 0-1
4	***A. tenuifolia***	18(78/93) 83.87%	36(5/93) 5.37%	17(6/93) 6.45%	16(3/93) 3.22%	-	-	-	(12/78)=12.9% 0-1
5	***A. tenuifoila***	18(24/34) 70.58%	36(2/34) 5.88%	17(3/34) 8.82%	16(4/34) 11.76%	-	19(1/34) 2.94%	(18/34)=52.94% 0-4B	(1/34)=2.94% 0-2
6	***A. tenuifoila***	18(18/30) 60%	36(4/30) 13.33%	17(2/30) 6.66%	16(3/30) 10%	-	19(3/30) 10%	-	-
7	***A. tenuifolia***	18(9/13) 69.23%	36(4/13) 30.76%	-	16(1/13) 7.69%	-	-	(1/13)=7.69% 0-1B	-
8	***A. tenuifoila***	18(26/40) 65%	36(7/40) 17.50%	17(2/40) 5%	16(3/40) 7.5%	15(2/40) 5%	-	(3/40)=7.5% 0-1B	(2/40)=5% 0-1
9	***A. tenuifoila***	18(47/52) 90.38	36(4/52) 7.69%	17 (1/52) 1.92%	-	-	-	(2/52)=3.84% 0-1B	-

To access the existence of published chromosome counts in the studied species, we used the most recent reports of chromosome numbers for this species (Chehregani and Mehanfar 2008; Chehregani and Hajisadeghian 2009), as well as the chromosome number databases, Index to Plant Chromosome Numbers (Missouri Botanical Garden, http://mobot.mobot.org/ W3T/Search/ipcn.html) and Index to Chromosome Numbers in the Asteraceae (Watanabe 2002, http:// www- asteraceae.cla.kobe-u.ac.jp/index.html).

Results

Nine populations of Achillea tenuifolia Lam and eight populations of A. bieberestinii Afan in Iran were studied in this research. Based on our results the basic chromosome number in the all studied populations was x=9, however, they were different regarding polyploid level from each other and two different ploidy level was determined in the studied populations. The results were represented as following.

Achillea tenuifolia

Nine populations of *Achillea tenuifolia* were studied for chromosome counting. In a population that was collected from Boghati mountain at the north of Hamedan province, chromosome number was 2n=18 (Fig.1A). A population with tetraploid chromosome number (2n=36) was also found in a protective area namely Lashkardar between the Malayer and Arak cities at the South of Hamedan provinve (Fig. 1B). In the all other studied populations that were collected from different regions of Hamedan and Kermanshah, two different chromosome numbers (2n=2x=18 and 2n=4x=36) were observed (Fig. 1 & Table 1). A population with just chromosome number 2n=2x=18 (Fig. 1a) and a population with chromosome number 2n=4x=36 (Fig. 1b) were determined, but the most common chromosome number among the seven remained populations is both 2n=18 and 2n=36 (Fig. 1 and Table 1). The chromosome number obtained for populations of A. tenuifolia (2n=2x=18) not supports the earlier report 2n=3x=27 by Khaniki (1995), but is accordance with chromosome number 2n=2x=18 reported by some prior researchers (Sheidai et al., 2009). While the chromosome number 2n=4x=36 is the first report for the species Achillea tenuifolia.

In addition to above-mentioned results, the study showed a regular aneuploid series 2n=15, 16, 17 and 19 within the some populations of this species (Table 2), that indicated occurrence conditions of aneuploidy in this species. In aneuploidy, reducing of chromosome number has been considered to be more common than increasing chromosome number since a descending change is simpler to produce than an ascending chromosome number.

B-chromosomes (Bs) were also observed in the some populations of this species (Fig. 1 and Table 2).

Table 3- Somatic chromosomes in the 8 population of *Achillea biberestinii* as detailed form.

Pop No.	Different Ch. number / Name of species	18	36	32	34	17	16	15	20	19	Number of B chromosome	Number of Satellite
1	***Achillea bieberestinii***	18 (9/9) 100%	-	-	-	-	-	-	-	-	(2/9)=22.22% 0-1B	(1/9)=11.11% 0-1
2	***A. bieberestinii***	18 (19/24) 79.16%	-	-	-	-	16(3/24) 12.5%	15 (1/24) 4.16%	-	19(1/24) 4.16%	(2/24)=8.33% 0-1B	(8/24)=33.33% 0-1
3	***A. bieberestinii***	18(17/25) 68%	36(2/25) 8%	-	-	-	16(3/25) 12%	-	20(3/25) 12%	-	(4/25)=16% 0-1B	(4/25)=16% 0-1
4	***A. bieberestinii***	18 (15/25) 60%	36(4/25) 16%	-	34(1/25) 4%	17(2/25) 8%	16(2/25) 8%	15(1/25) 4%	-	-	(1/25)=4% 0-2B	(3/25)=12% 0-2
5	***A. bieberestinii***	18 (42/51) 82.35%	36(3/51) 5.88%	32(1/51) 1.96%	-	-	16(5/51) 9.80%	-	-	-	(1/51)=1.96% 0-1B	(13/51)= 25.4% 0-1
6	***A. bieberestinii***	18 (27/36) 75%	36(3/36) 8.33%	-	34(4/36) 11.11%	-	16(2/36) 5.55%	-	-	-	(1/36)=2.77% 0-1B	(2/36)=5.55% 0-1
7	***A. bieberestinii***	18 (22/30) 53.33%	36(4/30) 13.33%	-	-	17(2/30) 6.66%	16(1/30) 3.33%	15(1/30) 3.33%	-	-	(4/30)=13.33% 0-1B	-
8	***A. bieberestinii***	18 (24/28) 85.71%	36(2/28) 7.14%	-	-	-	16(2/28) 7.14%	-	-	-	(1/28)=3.57% 0-1B	(12/28)=42.85% 0-2

The B-chromosomes were observed as much smaller than the A-chromosome , have a shape of round and did not pair with the A-chromosomes. B-chromosomes are accessory chromosomes existing in more than 1300 species of plants and 500 species of animals (Cammacho et al., 2000).

B-chromosomes (0-1) were reported in A. tenuifolia in a prior researche (Sheidai et al., 2009), while we were able to detect 0-4 B-chromosomes in the one population of this species. Variation in number of Bs among cells of the same root meristem was observed in some of the plants examined, which may be explained by nondisjunction during mitotic division of the meristem cells. Besides the variation in number from cell to cell and among populations, the B-chromosomes of A. tenuifolia diverged in size (from micro-size to about 0.7 µm) and morphology. The maximum number of cells containing Bs (52.94%) and the highest number Bs (0-4) were observed in the population collected from Soubashi in Hamedan province (see Fig. 1F).

Four populations of this species showed 0-1 satellaits chromosomes and one population have also 0-2 satellaits chromosomes. The population that has the highest number of cell contained satellaits chromosome (12.9%) collected from Assadbaad at the west of Hamedan province (Table 3).

Achillea biebersteinii

Chromosome counts were performed for the 8 populations of this species. Members of two populations, belonging to this species, showed chromosome number 2n=2x=18 (Fig. 2 A, B and Table 1).

One population was collected from Assadabad in the west of Hamedan province and other one collected from Ganjnameh area near the Hamedan city. In the other six populations two chromosome numbers (2n=2x=18 and 2n=4x=36) were observed at the same time (Fig. 2 and Table 1). Finding the chromosome number 2n=2x=18 confirmed the earlier reports (Morton 1981; Sheidai et al., 2009), and the chromosome number 2n=4x=36 for this species confirms the unique earlier report (Efimov 1998), but is opposite with the other report of chromosome number 2n=28 (Efimov 2005). Studies showed that polyploidy is common in this species, therefore existences of two ploidy levels for this species is accordance with prior reports. Otherwise we reported occurrence of B-chromosomes in this species for the first time that was 0-2 B-chromosomes in the most populations of this species. The maximum number of cells (22.22%) that contained Bs were observed in a population collected from Asadabad (Almagholagh montain) at the west of Hamedan province.

Only one population showed 0-2 Bs in their cells while other populations showed 0-1 Bs (Table 3).

In the otherwise the chromosome numbers of 2n=15, 16, 17, 19, 20, 32 and 34 were observed in the some populations (Table 3) that indicated the occurrence of aneuploidy in this species. The most occurrence of aneuploidy that were observed in this species were with chromosome numbers 2n=16 (12%) and 2n=20 (12%) in a population that collected from Ganjnameh at near the city of Hamedan.

In the most of populations of this species, 0-1 satellaits chromosomes were observed expect two populations that have 0-2 satellaits chromosomes. The population that has the highest number of cell contained satellaits chromosome (42.85%) collected from Nahavand (Gian forest) at the southwest of Hamedan province (see table 3).

Discussion

All of the examined populations have the same basic chro¬mosome number x=9 that is the most common basic number in tribe Anthemideae and the Asteraceae family (Inceer, et al. 2007). Polyploidy is another relevant evolutionary mechanism in plants (Wood, et al., 2009). Based on our results, few populations of the both species have chromosome number 2n=2x=18, and the most of populations of the species showed two different chromosome numbers 2n=2x=18 and 2n=4x=36 that suggests existence of different ploidy level among these species. In the same way, the polyploidy is also a response to the eco¬logical tolerances; It is suggested to study of ecological properties of the plant habitats. Chromosome number x=9 is the primitive basic chromosome number from which the others have been derived by aneuploidy (2n=15, 16, 17, 19, 20, 32 and 34) and autoploidy 2n=4x=36. Among the considered populations, most of populations of both species lose or received single or multiple chromosomes that is represented existence of aneuploidy in the genus. Aneuploidy arises from meiotic or mitotic deviations, or radiation and chemical treatment response (Stebbins 1971) and probably played an important role in the speciation mechanism and seeming to be an important component of chromosomal evolution in this genus. The presence of B-chromosomes, which is common in the species of A. tenuifolia and A. bieberestinii, is one of the most particular futures of the both species. Meristem cells showed variation in B-chromosome numbers, size and morphology, both in individual cells of the same plant and in different plants.

Acknowledgement

This research was done using a grant provided by research council of Bu-Ali Sina University.

References

1- Androschchuk A. F. and Kostinenko L. D., (1981) Chromosome numbers of the genus *Achillea L.* Certain species cultivated in botanical gardens. Ukrajinsk Bot. Zurn. (Kiev), 38: 53-57.
2- Benedek B. and Kopp B. (2007) Achillea millefolium L. s. l. revisited: recent findings confirm the traditional use. Wien Med Wochenschr. 157: 312-314
3- Bremer K. and Humphries C. J. (1993) Generic monograph of the *Asteraceae-Anthemideae*. Bull. Nat. Hist. Mus. London (Bot.) 23: 71-177.
4- Camacho J. P. M., Sharbel T. F., and Beukeboon L. W. (2000) B-chromosome evolution. Philos Trans R Soc Lond B Biol Sci 355:163–178
5- Carr G. D., King R. M., Powell A. M. and Robinson H. (1999) Chromosome number in Compositeae XVIII. Am. Jour. Bot. 86: 1003-1013.
6- Chehregani A. and Mehanfar N. (2008) New Chromosome Counts in the Tribe Anthemideae (Asteraceae) from Iran. Cytologia 73: 189-196.
7- Chehregani A. and Hajisadeghian S. (2009) New chromosome counts in some species of Asteraceae from Iran. Nordic Jour. Bot. 27: 247-250.
8- Chehregani A., Atri M., Yousefi S. and Jalali F. (2010) Polyploidy variation in some species of the genus *Artemisia L.* (Asteraceae) in Iran. Caryologia 63: 168-175.
9- Chehregani A., Mohsenzaeh F., Moazen F. (2012) Variation of chromosome numbers in Tanacetum parthenium Schultz Bip. (Asteraceae) in Iran. Chromosome Botany 7: 97-100.
10- Chehregani A., Heidarian M., Jalali F. (2013) Cytological investigations on *Achillea wilhelmsii* C. Koch (Asteraceae) in the western part of Iran. Chromosome Botany, 8:13-16.
11- Contandriopoulos J. and Martin D., (1967) Contribution a I'etude cytotaxonomique des Achillea de Grece. Irregularite de la meiose. Bull. Bot. Soc. France, 114: 257-275.

12- Constantinidis T. H. and Kalpotzakis E., (2005) A new species of *Achillea L.* (Asteraceae: Anthemideae) from South-East Peloponnisos, Greece. Bot. J.Linn. Soc. 147: 249–256.
13- D˛abrowska J., 1989. The chromosome number of several taxa of the genus *Achillea L.* Acta Soc. Bot. Poloniae 58: 163-177.
14- D˛abrowska J. (1992) The chromosome number and DNA content in taxa of *Achillea L.* In relation to the distribution of the genus. Acta Universitatis Wratislaviensis No. 1425, Prace Botaniczne 49: 1–84.
15- Danihelka J. and Rotreklov O., (2001) Chromosome numbers within the *Achillea millefolium* and the *A. distans* groups in the Czech Republic and Slovakia. Folia Geobot. 36: 163–191.
16- Danihelka J. and Rotreklov O., (2002) *Achillea distans* (Asteraceae) confirmed as native in the Bieszcady Mts (SE Poland). Fragmenta Floristica et Geobotanica 45 (2000): 257–263.
17- Dyer A. F., Jong K., Ratter J. A. (1970) Aneuploidy: a redefinition. Notes from the Royal Botanic Garden Edinburgh 30: 177-182.
18- Efimov K. F., (1998) Chromosome numbers in some species of the families Asteraceae, Campanulaceae, Fabaceae from central Caucasus. Bot. Žurn. (Moscow & Leningrad) 83(8): 131–132.
19- Efimov K. F., (2005) Chromosome numbers of some species of the families Amaryllidaceae, Asteraceae, Fabaceae in North Osetia. Pages 33-35 in Karyology, Karyosystematics and Molecular Phylogeny. St. Petersburg, Russia.
20- Ehrendorefer F., and Guo Y.P. (2006) Multidisciplinary studies on *Achillea sensu* lato (Compositae –Antemideae): new data on systematic and phylogeography Willdenowia 36: 69-87.
21- Funk V. A., Susanna A., Steussy T. F., and Bayer R. J. (2009) Systematics, evolution and biogeography of Compositae, International Association for Plant Taxonomy.
22- Ghaffari S. M. and Kelich K. (2006) New or rare chromosome counts of some angiosperm species from Iran. Iran. Jour. Bot. 12: 81-86.
23- Goli S. A. H., Rahimmalek M., Sayed Tabatabaei B. E. (2008) Characteristics and fatty acid profile of yarrow (*Achillea tenuifolia*) seed oil. Int. J. Agric. Biol. 10, 355–357.
24- Guo Y. P., Ehrendorfer F., and Samuel R. (2004) Phylogeny and systematics of *Achillea* (Asteraceae-Antemideae) inferred from nrITS and plastid trnL-F DNA sequences. Taxon 53: 657-672
25- Guo Y. P., Saukel J., Mittermayr R., Ehrendorfer F. (2005) AFLP analyses demonstrate genetic divergence, hybridization, and multiple polyploidization in the evolution of *Achillea* (Asteraceae – Antemideae). New phytol 166: 273-289.
26- Halliday G., and Beadle M. (1980) Flora Europaea. Volume: 5, Cambridge, London.
27- Inceer H., and Hayirlioglu-Ayaz S. (2007) Chromosome numbers in the tribe Anthemideae (Asteraceae) from north-east Anatolia. Botanical Journal of the Linnean Society, 153: 203–211.
28- Karamenderes C., and Apaydin S. (2003) Antispasmodic effect of *Achillea nobilis L.* subsp. sipylea (O. Schwarz) Bässler on the rat isolated duodenum. Journal of Ethenopharmacology 84:175-179.
29- Khaniki G. B. (1995) Chromosome numbers and morphometry in Achillea (Anthemideae, Compositae). Nucleus 38: 104-111.
30- Lawrence W. E. (1947) Chromosome numbers in *Achillea L.* in relation to geographic distribution. Amer. J. Bot. 34: 538–545.
31- Leitch I. J. and Bennett M. D. (1997) Polyploidy in angiosperms. Trends in Plant Science 12: 470-476.
32- Liove F. (1972) IOPB Chromosome number reports. Taxon 21: 333–346.
33- Liove F. (1973) IOPB Chromosome number reports. Taxon 22: 285–291.
34- Maffei M., Germano F., Doglia G. and Chialva F. (1993) Essential oils, chromosome numbers and karyotypes from Italian *Achillea L.* species. Part II. J. Ess. Oil Res. 5: 61–67.
35- Magulaev A. J. (1982) The number of chromosomes of the species of Asteraceae, Caryophyllaceae and Plantaginaceae of the North Caucasus. Sci. Rep. Higher. School Biological Science, 11: 74-79.
36- Moore D. M. (1982) Flora Europaea check-list and chromosome index. 423 S., Cambridge University press, Cambridge. UK.

37- Morton J. K. (1981) Chromosome numbers in Compositae from Canada and the U.S.A. Bot J Lin Soci 82:357-368.
38- Mozaffarian V. (2005) Notes on the tribe Anthemideae (Compositae), new species, new records and new combination for Iran. Iran. Jour. Bot., 11: 115-127
39- Mozaffarian V. (2007) A Dictionary of Iranian Plant Names, Farhang Moaser Publishers. Tehran, Iran.
40- Oswiecimska M. (1974) Korrelation zwischen Chromosomenzahl und Prochamazulenen in Achillea von Osteuropa. Planta Med., 25: 389-395.
41- Podlech D. (1986) Compositeae VI. Antemideae. In: Flora Iranica, ed. Rechinger K.H., NO.158., pp 49-71. Skademische Druck-u., Verlagsans Talt,Graz, Austria.
42- Post G. E. (1933) Flora of Syria, Palestina and Sinai. American Press, Beirut 2: 44-47.
43- Rahimmalek M., Sayed Tabatabaei B. E., Arzani A. and Etemadi N. (2009) Assessment of genetic diversity among and within Achillea species using amplified fragment length polymorphism (AFLP). Bioch. Syst. Ecol. 37: 354–361.
44- Saukel J., Anchev M., Guo Y. P., Vitkova A., Nedelcheva A., Goranova A., Konakchiev A., Lambrou M., Nejati S., Rauchensteiner F., Ehrendorfer F. (2004) Comments on the biosystematics of Achillea (Asteraceae –Anthemideae) in Bulgaria. Phytol Balcanica, 9: 361-400.
45- Sheidai M., Azanei N. and Attar F. (2009) New chromosome number and unreduced pollen formation in Achillea species (Asteraceae). Acta Biologica Szegediensis. 53:39-43.
46- Soltis P. S. and Soltis D. E. (2000) The role of genetic and genomic attributes in the success of polyploids. Proc. Natl. Acad. Sci. USA, 97: 7051-7057.
47- Soltis D. E., Soltis P. S. and Tate J. A. (2003) Advances in the study of polyploidy since Plant speciation. New Phytologist, 161: 173–191.
48- Stebbins G. L. (1971) Chromosomal evolution in higher plants. Arnold, London.
49- Tutin T. G., Halliday G. and Beadle M. (1980) Flora Europaea. Vol. 5. Cambridge University Press, Cambridge.
50- Vallès J., Garnatje T., Garcia S., Sanz M. and Korobkov A. A. (2005) Chromosome numbers in the tribes Anthemideae and Inuleae (Asteraceae). Kazakhstan. Bot. J. Lin. Soc. 148: 77-85.
51- Watanabe K. (2002) Index to chromosome numbers in Asteraceae. http://www-asteraceae.cla.kobe-u.ac.jp/ index.html.
52- Wendel J. and Doyle J. (2005) Polyploidy and evolution in plants. In: Henry R J (ed.) Plant diversity and evolution: genotypic and phenotypic variation in higher plants. pp. 97-117. Wallingford, CABI Press.
53- Wendel J. F. (2000) Genome evolution in polyploids. Plant Molecular Biology 42: 225–249
54- Wood T. E., Takebayashi N., Barker M. S., Mayrose I., Greenspoon Ph.B. and Rieseberg L. H. (2009) The frequency of polyploid speciation in vascular plants. Proc Natl Acad Sci USA106: 13875-13879.
55- Zargari A. (1996) Medicinal Plants. Tehran University Publication; 4th ed. Vol. 3,. pp. 106-117, Tehran, Iran.
56- Zheng-Yi W., and Raven P. H. (1994) Flora of China. Vol. 17. Science Press, Missouri Botanical Garden, USA.

Research Article

Karyological study of the Caspian bent-toed Gecko *Cyrtopodion caspium* (Sauria: Gekkonidae) from North and North-Eastern of Iran

Farahnaz Molavi[1*], Haji-Gholi Kami[2], Morteza Yazdanpanahi[1]

1-Department of Biology, Faculty of sciences, Mashhad Branch, Islamic Azad University, Mashhad, Iran
2-Department of Biology, Faculty of Sciences, Golestan University, Gorgan , Iran

Received 21 January 2014 Accepted 01 March 2014

Abstract

The diploid number among Gekkonid lizards ranges from 2n=16 to 2n=46. The majority of geckos' chromosomes are small which makes the study of their chromosomes very difficult. For this reason, karyotype of *Cyrtopodion caspium* and some similar gecko are still not known. The close relatives of *C. caspium* have variation in the chromosomal number in their populations. So to find out possible variability among these populations in Iran. We described the karyotype of testis and bone marrow of 14 specimens of this species from seven provinces and 10 localities in north and north-eastern parts of Iran in 2011. They showed 2n=38 for all populations. There is not intraspecific chromosome polymorphism in *Cyrtopodion caspium*. The karyotype consists of one pair of metacentric element and 17 pairs of telocentric or subtelocentric elements and one pair of three arm chromosomes. The number of haploid chromosomes was 21 for three male specimens. The chromatid separation did not show heteromorphic sex chromosomes. Our results highlight the need for continued research into the basic biology and taxonomy of *Cyrtopodion caspium* in Iran.

Keywords: Chromosome structure, Karyology, Sex chromosome, Lizard, Iran, and Caspian bent toed gecko

Introduction

The family Gekkonidae is the most diverse and the oldest group of reptiles which have a worldwide distribution (Anderson, 1999). It is the largest family of lizards, comprising 100 genera and 943 species. Although there are several studies on this species, a few of them are related to the karyotype of this family (Ahmadzadeh *et al.*, 2004, 2005, 2008; Hojati *et al.*, 2009).

Cyrtopodion caspium is a small sized oviparous nocturnal lizard, as a house gecko, it is distributed in many cities and villages in Iran. It consists of two subspecies, *Cyrtopodion caspium caspium* (Eichwald, 1831) and *Cyrtopodion caspium insularis* (Akhmedov and Szczerbak, 1978). The main habitats of *C. c. caspium* in Iran are located in the Gorgan region of Mazandaran, to northern and eastern Khorasan, extending south to Sistan and west to Azerbaijan. *C. c. insularis* has only been reported from a limited region in the Caspian Sea. (Rastegar-Pouyani *et al.*, 2008; Rhodin *et al.*, 2010).

The diploid number among Gekkonid lizards ranges from 2n=16 to 2n=46 (Schmidt *et al.*, 1994).The typical karyotype consists of a gradual series of acrocentric chromosomes which there is no difference between macro and micro chromosomes.

The sex determination mechanisms in Saurian have not been completely understood. Sex chromosome evolution in recent data from some lizard's families suggests that they have multiple origins (Beak, 1983). There are species with chromosomal sex determination mechanisms ascribed to male heterogamete in the family's Iguanidae (Frost & Etheridge, 1989), Lacertidae, Teiidae, Scincidae, and Pygopodidae. Female heterogamety is known in the family's Gekkonidae, Varanidae, and Lacertidae (Peccinini-Seale *et al.*, 1981). In the genus *Cnemidophorus*, Cole *et al.* (1969) and Bull (1978) reported a chromosomal sex determination mechanism of the type XX: XY for *Cnemidophorus tigris*. We performed a comparative karyological analysis among different populations of *Cyrtopodion caspium*, from North and North-Eastern Iran, in order to find out possible variability among these populations.

∗Corresponding author E-mail:
fm_yazdan@yahoo.com

Material and Methods

During the field work from May to June 2011, we have collected 14 adult specimens (9 males and 5 females) of *Cyrtopodion caspium* from seven provinces and 10 localities of north and north-eastern of Iran (Table 1). All individuals of these species were transferred to the laboratory at the IAU branch of Mashhad, and their karyotypes were determined from all specimens.

Chromosomal preparations were obtained from suspension of bone marrow cells from femur and vertebral column and of spleen cells according to the procedures of Porter and Sites (1986). Meiotic chromosomal spreads were prepared from testis suspension adapted for lizards by Peccinini-Seale *et al*. (1971). Metaphase plates were obtained by the squash method, with treatment of vinblastine specimens. For 9 male individuals at least 11-21 complete metaphases were analyzed. Slides were stained by 4% gimsa with pH 7.2. The photographs were taken on a Zeiss microscope with 100X magnification. Chromosome number and properties were determined by MIP Software.

Table 1.Sampling localities of *Cyrtopodion caspium*

	Locality- province	**Longitude**	**Latitude**	**Altitude (m)**
1	Anzali port- Gilan	49 º 25/	37 º 28/	15
2	Siyahdarreh- Gilan	49 º 08/	37 º 07/	10
3	Fereydounkenar-Mazandaran	53 º 23/	36 º 50/	-10
4	Zaghmarz- Mazandaran	53 º 33/	36 º 43/	-10
5	Ziyarat village-Golestan	54 º 24/	36 º 54/	250
6	Alagol wetland- Golestan	54 º 36/	37 º 17/	00
7	Kalat- Northern Khorasan	59 º 40/	37 º 00/	1000
8	Ahmad Abad village – Semnan	56º 42/	35º 48/	1000
9	Shandiz – Razavi Khorosan	59 º 25/	33 º 56/	1360
10	Byrjand - Southern Khorasan	59 º 13/	32 º 53/	1491

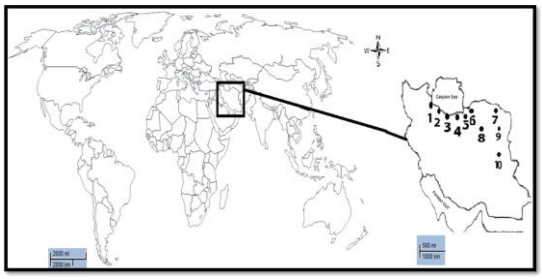

Figure 1.The map of sampling localities.

Results

The karyotype of *Cyrtopodion caspium* is shown in figure 2. Metaphase analyses showed a diploid number of 38 chromosomes (2n=38). The fifth pair was metacentric and other pairs were sub-telocentric or telocentric, and there is a pair with three chromatids. Macro and microchromosomes were indistinguishable and no sex chromosome polymorphism could be observed (Figure 1and Table2 and 3).

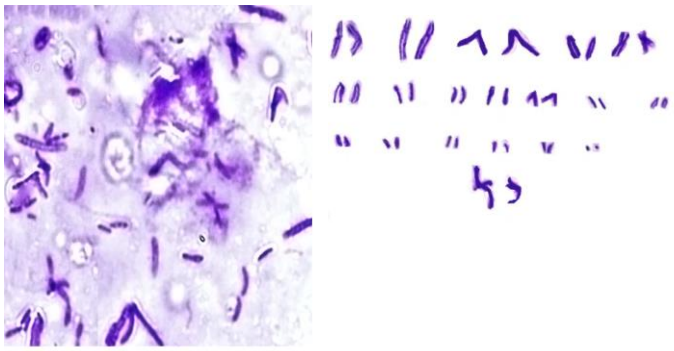

Figure 2. Karyotype of Cyrtopodion caspium.

Table 3. Calculations on karyotype of Cyrtopodion caspium.

Order	**Variable name**	**Value**
1	2n	38
2	Number of Arms	46
3	Fn	44
4	Fna	2
5	All Chromosome Lenght	1490.08 µm
6	Total Metacentric	0
7	Total Submetacentric	8
8	Total Acrocentric	24
9	Total Telocentric	6

No heteromorphic sex chromosomes were observed in the karyotype of Cyrtopodion caspium. Meiotic analysis showed a haploid number of 21 chromosomes (n=21) for male specimens (Figure 3).

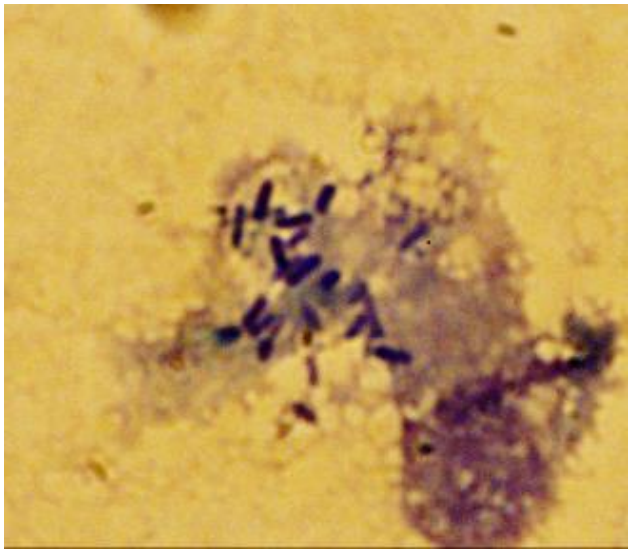

Figure 3.Karyotype of spermatid cell in *C. caspium.*

There was variability in number (1-2 nucleoli) and size (small, medium and large) of nucleoli in the 50 analyzed interphase cells (Figure 4).

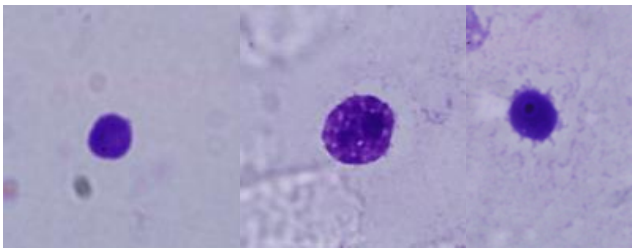

Figure 4.Interphasic nuclei of bone marrow cells.

However, T.F-factor that stands for symmetry is lower than 50% and shows that chromosomes are not in symmetrical position and are located very far from chromosomal symmetry.

$$T.F\% = \frac{\text{Short arms lengths}}{\text{Total arms lengths}} \times 100$$

T.F. = 1.594

Moreover, to show the symmetrical position the DRL index is employed whichit's amaximum relative length of chromosomes minus from minimum relative length of chromosomes. DRL index equals 0.1069 that confirms lack of symmetrical condition in the species.

Table 2. Description of Cyrtopodion caspium chromosomes.

No.	Length (µm)	Up Arm (µm)	Down Arm (µm)	Lcn/L total	Centromer Index (µm)	Chromosome Type
1	93.66	24.6	69.07	0.063	0.26	Acrocentric
2	76.48	29.07	47.41	0.051	0.38	Acrocentric
3	72.47	33.06	39.41	0.049	0.46	Sub-metacentric
4	74.09	25.08	49.01	0.05	0.34	Sub-metacentric
5	83.41	5.39	78.03	0.056	0.06	Telocentric
6	80.35	6.32	74.03	0.054	0.08	Telocentric
7	50.18	5	45.18	0.034	0.1	Acrocentric
8	50.43	5.83	44.6	0.034	0.12	Acrocentric
9	43.05	5.1	37.95	0.029	0.12	Telocentric
10	47.01	5	42.01	0.032	0.11	Telocentric
11	35.45	12.04	23.41	0.024	0.34	Submetacentric
12	18.3	8.25	10.05	0.012	0.45	Submetacentric
13	40.54	5.1	35.44	0.027	0.13	Telocentric
14	41	5.1	35.9	0.028	0.12	Telocentric
15	34.96	10.44	24.52	0.024	0.3	Submetacentric
16	37.25	12.17	25.08	0.025	0.33	Submetacentric
17	33.14	9.06	24.08	0.022	0.27	Acrocentric
18	35.06	12.04	23.02	0.024	0.34	Acrocentric
19	31	5	26	0.021	0.16	Acrocentric
20	39.11	5.1	34.01	0.026	0.13	Acrocentric
21	26.49	9.49	17	0.018	0.36	Submetacentric
22	32.7	7.07	25.63	0.022	0.22	Submetacentric
23	26	5	21	0.017	0.19	Acrocentric
24	24.41	5.1	19.31	0.016	0.21	Acrocentric
25	22.36	6.08	16.28	0.015	0.27	Acrocentric

26	22.19	5.1	17.09	0.015	0.23	Acrocentric
27	21	5	16	0.014	0.24	Acrocentric
28	20.96	5.83	15.13	0.014	0.28	Acrocentric
29	18.57	6.4	12.17	0.012	0.34	Acrocentric
30	19.66	8	11.66	0.013	0.41	Acrocentric
31	18.73	5.39	13.34	0.013	0.29	Acrocentric
32	19	5	14	0.013	0.26	Acrocentric
33	18.34	5	13.34	0.012	0.27	Acrocentric
34	16.67	7.62	9.06	0.011	0.46	Acrocentric
35	12.22	3	9.22	0.008	0.25	Acrocentric
36	9.06	0	9.06	0.006	0	Acrocentric
37	59.74	5	54.74	0.04	0.08	Acrocentric
38	85.04	7.81	77.23	0.057	0.09	Acrocentric

Discussion

Chromosomal evolution has been occurred in all vertebrates. The results of other survey reveal that many minor chromosome rearrangements have been occurred. However, minor deletions throughout the genome of the ancestors of reptiles and birds have led to reduce birds' genome to 50% of reptiles 'genome (Swanson *et al.*1981; Edwards 2009; Iturra*et al.,* 1994).

Single or multiple centric fissions are the main chromosome rearrangement found in the evolution of lizard karyotype, including *Anolis*, *Scelopurus grammicus* and *Liolaemus* (Webster *et al.,* 1972; Lamborot andAlvares-Sarret,1989; Sites, 1983; Lamborot, 1991)

There is not intraspecific chromosome polymorphism in *Cyrtopodion caspium*, which have not shown considerable chromosome variations in several examined populations. Although Intra-specific chromosomal polymorphism is generally between 10-50 % in lizards (Iturra*et al.,* 19940).

Some authors have argued that the 2n=38 is ancestral for the family Gekkonidae (and perhaps the entire suborder), although others have expressed different opinions (Shibaike *et al.,* 2009; Oliver *et al.,* 2007).

However all specimens from seven provinces were monomorphism. The diploid number of 2n=38 found in *Cyrtopodion caspium* is situated within the range of the family.

Different specimens of *Diplodactylus tessellatus* from Australia exhibit 2n=28, 30, and 38 that 2n= 38 is the ancestral case. 38 chromosomes are reduced to 30 and 28 through minor fissions of some acrocentric chromosomes. Moreover, 2n=38, 36, 34, 30 are reported from *Vittatus diplodactylus* (Oliver et *al*., 2007).

In *Tarento lamauritanica* (Gekkonidae) only one chromosome number has been reported (2n=42). Many herpetologists believe that more studies need to investigate variation in chromosome number (Shibaike *et al.,* 2009 and Hidestonhi*et al.*2001). For example, many studies on *Diplodactylus vittatus* show high variation in chromosome number in members of Gekkonidae. The above species exhibits 3 different chromosome numbers from different regions. (King 1977) Geckoes' karyotypes are 2n=38 in many cases. However, diversity in chromosome numbers has been observed in geckos. It should be emphasize that different chromosome numbers are available in populations which are similar morphologically. (Shibaike *et al.,* 2009).

Gekko gecko (one of the near relatives of *C. caspium*) exhibits only 2n=38 and there is no variation in chromosome number. However, it should be considered that only one specimen of G. gecko has been investigated to study the karyotype of this species. (Cohen *etal.*1967)

Evolution of karyotypes occurs in neighboring geographical regions; for example, chromosome evolution of 9 Gekkonidae species are reported from Eastern Asian islands (*G. shibatai, G. tawaensis, G. vertebral* is, *G. yakuensis*, and 3 non-described species). Moreover the number of chromosomes are 2n=38 in all of specimens. However different karyotypes have been investigated from different populations (Shitake and Takahashi, 2009).

There are many acrocentric chromosomes in all of reptiles (Hidetoshi *et al.,* 2001). The majority of geckoes' chromosomes are small which makes the study of their chromosomes very difficult (Katia andMachado, 1995).

Karyotypes with no distinction between macro and micro chromosomes, as the one we found in *C. caspium* are also typical of the Gekkonidae.

Heteromorphic sex chromosomes were not observed in *C. caspium.* It was corroborated by the absence of a distinctive heteromorphic bivalent in male cells. The presence of heteromorphic sex chromosome in Gekkonidae is known only from 10 species belonging to five genera (Shitake and Takahashi, 2009).However as far as we know; there is no reference between sexual chromosomes in *Cyrtopodion*. (Katia andMachado, 1995)

Most Gekkonids have a chromosomal mechanism

of sex determination of the ZZ:ZW type (Kawai *et al*., 2009) , in which the heterogametic sex is the female, such as in *Heteronotia binoei* (Mortiz,1990), *Gehyra australis* (King 1983), *Gehyra purpuracens* (Moritz,1984) and *Cyrtodactylus pudisulcus* (Shitake and Takahashi 2009). But, in a few species, the mechanism is of the XX: XY type, such as in *Gekko gecko* (Solleder and Schmid 1984), *Gekko japonica* (Mortis 1990) and *Gonato desceciliae* (McBee*et al.,* 1987). *G. hokouensis* demonstrates high diversity of sex chromosomes and their rapid evolution; however, mechanisms of sex determination are evolved rapidly (Ezaz *et al.*, 2009).
Temperature is the most important factor in determination of sex in lizards ,for example ancestral Lephodosaur (Janes*et al.,* 2009) but in some cases sex chromosomes determine gender (Janes *et al.,* 2009). For example, there are sex chromosomes in *Gekko hokouennsis* and sex determination is not related to environmental factors in these species (Kawai *et al.,* 2009).

Acknowledgments

We appreciate the efforts of Narges Tehrani, who assisted in the collection of the lizards. Special thanks also to Dr. Darvish, Because He was allowed to use the MIP software. This software is limited to Rodentology Research Department (RRD).

References

1. Ahmadzadeh F. (2004) A preliminary study of the Lizard fauna and their habitats in Meshkinshahr district. Environmental Sciences. 1(2). 39-44.
2. Ahmadzadeh F., Kiabi B. H., Kami H. G.,and V. Hojati. (2008) A preliminary study of the Lizard fauna and their habitats in Northwestern Iran. Asiatic Herpetological Research. Vol 11, pp. 1-9.
3. Ahmadzadeh F., Kiabi B. H., Zehzad B., and Kami H. G. (2005) A survey on Lizard fauna in the northern part of Ardebil province of Iran. First international conference of biology. 23-25 August. GilanUniversity. Rasht. Iran.
4. Anderson S. C. (1999) The lizards of Iran. Contributions to Herpetology. Vol. 15, Society for the Study of Amphibians and Reptiles, Saint Louis, Missouri: i-vii, 35-44.
5. Beçak W. (1983) Evolution and differentiation of sex chromosome in lower vertebrates. Differentiation, 23(supl.): 3-12.
6. Bull J. J. (1978) Sex chromosome differentiation: an intermediate stage in a lizard. Canadian Journal of Genetics and Cytology, 20: 205-209.
7. Cohen M. M., Huang Ch., and Clrak H. F.. (1967) The somatic chromosomes of 3 lizard species: *Gekko gecko*, *Iguana iguana*, and *Crotaphytus collaris* cellular and molecular life sciences. Vol. 23, No. 9,769-771
8. Cole C. J., Lowe C. H., and Wright J. W. (1969) Sex Chromosomes in Teiid Whiptail Lizards (Genus *Cnemidophorus*). American Museum Novitates, 2395: 1-14.
9. Eric J. V., and Bruce T. L. (2006) Multiple independent origins of sex chromosomes in amniotes PNAS. 103(20): 18031 –18048
10. Ezaz T., Quinn A. E., Sarre S. D., O'Meally D., Georges A., Marshall J. A., and Graves M.. (2009) Molecular marker suggests rapid changes of sex-determining mechanisms in Australian dragon lizards ,Chromosome Research , Vol.17, 91-98.
11. Frost D. R., and Etheridge R. (1989) A phylogenetic analysis and taxonomy of iguanian lizards (Reptilia: Squamata). Misc. Publ. Mus. Nat. Hist. Univ. Kansas, 81: 1-65.
12. Hidetoshi O., Hikida T., Nabhitabhata J., and Panha S.. (2001) Cryptic Taxonomic Diversity in Two Broadly Distributed Lizards of Thailand (*Mabuya macularia* and *Dixonius siamensis*) as Revealed by Chromosomal Investigations (Reptilia: Lacertilia) The Natural History Journal of Chulalongkorn University. 1(1): 1-7.
13. Hojati V., Kami H. G., Faghiri A., and Ahmadzaheh F. (2009) A morphological study of Bedriaga plate- tailed *Gecko*, *Teratoscincus bedriaga* Nikolsky 1900, in Semnan province of Iran (Reptilia: Gekkonidae), Zoology in the Middle East. 46: 113-115.
14. Iturra P., Veloso A., Espejo P., and Navarro J. (1994) Karyotypic and meiotic evidence for a Robertsonian chromosome polymorphism in the lizard *Liolaemus fuscus* (Tropiduridae, Sauria). Brazilia Journal of Genetics. 17(2): 171-174.
15. Janes, D. E., Ezaz T., Graves J. A. M., and Edwards S. V. (2009) Recombination and nucleotide diversity in the pseudoautosomal region of minimally

differentiated sex chromosomes in the Emu, *Dromaius novaehollandiae*. Journal of Heredity 100(2): 125-136.
16. Katia C., and Machado P. (1995) C-and R-banding patterns and nucleolus organizer regions in the karyotype of *Hemidactylus mabouia* (Sauria, Gekkonidae). Brazilia Journal of Genetics. 18, p. 527-531.
17. Kawai A., Junko I., Nishida C., Ayumi K., and Ota H.. (2009) The ZW sex chromosomes of *Gekko hokouensis*(Gekkonidae, Squamata) represent highly conserved homology with those of avian species. Chromosoma 118: 51-43.
18. King M. (1977) Chromosomal and morphometric variation in the *GekkoDiplodactylus vittatus*. Austerallian Journal of Zoology 25(1): 57-43
19. King M. (1983) Katyotypic evolution in *Gehyra* (Gekkonidae: Reptilia). III. The *Gehyra australis* complex. Aust. J. zool. 31: 723-741.
20. Klug M. (1991) Checklist of gekkonoid lizards. Smithsonian Herpetological Information Service 85: 1-35.
21. Lamborot M., and Alvares-Sarret E. (1989) Karyotypic characterization of some *Liolaemus* lizards in Chile (Iguanidae). Genome. 32: 393-403.
22. Lamborot, M. (1991) Karyotypic variation among populations of *Liolaemus monticola* (Tropiduridae) separated by riverine barriers in the Andean Range. Copeia (4): 1044-1059.
23. Levan A., Fredga K., and Sandberg A. A. (1964) Nomenclature for centromeric position on chromosomes. Hereditas, 52(2): 201-220.
24. McBee K., Bickham J. W., and Dixon J. R. (1987) Male heterogamy and chromosomal variation in Caribbean geckos. Journal of Herpetology 21 (1): 68-71.
25. Mortiz C. (1984) The evolution of a highly variable sex chromosome in *Gehyra purpurascens* (Gekkonidae): Chromosome 90: 111-119.
26. Mortiz C. (1990) Patterns and processes of sex chromosome evolution in Gekkonid lizards (Reptilia: Sauria). In Cytogenetics of Amphibians and Reptiles (Olmo, E., Ed.). BirkhauserVerlag, Switzerland, pp. 205-220.
27. Oliver P. M., Hutchinson M. and Hutchinson R. (2007) Karyotypic variation in the Australian gecko *Ciplodactylus tessellatus*, with the description of a new karyotypic complement for diplodactyline geckos Journal of Herpetology 41 (3): 540-543.
28. Peccinini-Seale D. (1981) New developments in vertebrate cytotaxonomy. IV. Cytogenetic studies in reptiles. Genetica, 56: 123-148.
29. Peccinini-Seale D., and Frota-Pessoa O. (1974) Structural heterozygozyty in parthenogenetic populations *of Cnemidophorus lemniscatus* (Sauria: Teiidae) from the Amazonas Valley. Chromosome, 47: 439-451.
30. Porter C. A., and Sites J. W. (1986) Evolution of *Sceloporus grammicus* complex (Sauria: Iguanidae) in central Mexico: population cytogenetics. Syst. Zool. 35: 334-358.
31. Rastegar-Pouyani N., Kami H. G., Rajabzadeh M., Shafiei S., and Anderson S. C. (2008) Annotated check list of amphibians and reptiles of Iran. Iranian Journal of Animal Biosystematics(IJAB). 4 (1), 43-66.
32. Rhodin A. G. J., Dijk P. P. V., Iverson J. B., Shaffer H. B. (2010) Turtle of the world: Annotated check list of taxonomy, synonymy, distribution and conservation Status. Chelonian Research Monographs. (5). 85-164.
33. Schmid M., Feichtinger W., Nanda I., Schakowski R., Garcia R. V., Puppo J. M., and Badillo A. F. (1994) An extraordinary low diploid chromosome number in the reptile *Gonatodestaniae* (Squamata, Gekkonidae). J. Heredity. 85: 255-260.
34. Scott C. C., and Edwards V. (2009) Genome evolution in Reptilia: in silico chicken mapping of 12,000 BAC-endsequences from two reptiles and a basal bird, Proceedings of the Avian Genomics Conference and Gene Ontology Annotation Workshop.
35. Shibaike, Y., Takahashi Y., Arikura I., Iiizumi R., Kitakawa S., Sakai M., Imaoka C., Shiro H., Tanaka H., Akakubo N., Nakano M., Watanabe M., Ohne K., Kubota S., Kohno S., and Ota H. (2009) Chromosome evolution in the lizard genus *Gekko* (Gekkonidae, Squamata, Reptilia) in the East Asian islands. Cytogenetic and Genome Research.127:182-190.
36. Sites J. W. (1983) Chromosome evolution in the iguanid lizard *Sceloporus*

grammicus. Chromosome polymorphisms. Evolution 37: 38-53.
37. Swanson C. P., Merz T., and William J. A. Y. (1981) Cytogenetics: The chromosome in division, Inheritance and Evolution. 2nd ed. Englewood Cliffs, N.J.: Prentice-Hall. xiv, 577pp.
38. Webster T. P., Hall W. P., and Williams E. E. (1972) Fission in the evolution of a lizard karyotype. Science. 77: 611-613.

Research Article

Paternal genetic affinity between Iranian Azeris and neighboring populations

Ardeshir Bahmanimehr[1,3*] and Fatemeh Nikmanesh[2]

1. *Biotechnology Department, Marvdasht branch, Islamic Azad University, Marvdasht, Iran*
2. *Biotechnology and Bioengineering Department, School of Advanced Medical Science, Tabriz University of Medical Sciences, Tabriz, Iran.*
3. *Institute of Molecular Biology, National Academy of Sciences of Armenia, 07 Hasratian St.,Yerevan, Armenia*

Received 06 Feb 2014 Accepted 18 March 2014

Abstract

In certain environments such as Iran highlands, major innovations in lifestyle, as the emergence of agriculture and domestication of animals, are thought to have led to population expansions. Historical studies showed that at some point in history (from the third to the first millennium BC) dramatic changes have been taken place on the Iranian plateau. To trace the genetic affinity between the Iranian Azeris and neighboring populations, 297 samples were collected from northwest of Iran. The non-recombining portion of the Y chromosome (NRY) was genotyped at the unique event polymorphism (UEP) levels, using 48 single-nucleotide polymorphism (SNP) markers, based on the human NRY tree. According to our results, like other Iranian ethnic groups, Iranian Azeris showed a heterogeneous paternal genetic structure. Low genetic distances were also found between Iranian Azeris and their contemporary geographical neighbors. They also have preserved minor share of Y-haplogroup of central Asian ancestry tracts in their genomes, which is in agreement with the historical period of major Turkic migrations. The strategic feature for northwest of Iran to transfer and carry important ancient migratory events and gene flow across the Asia and the Europe also conducive conditions for sedentary habitation leading to sharp demographic growth in the area is supported by all molecular and statistical analysis of this study.

***Keywords*:** Iranians; Y-chromosome genomes; phylogeny; population expansions; genetic history

Introduction

The modern and interdisciplinary science, Molecular anthropology, is defined as the use of molecular genetics techniques encompasses the analysis and interpretation of; molecular genetic variation in various patterns contemporary human populations that anthropologists are interested in concerning human evolution and diversity. By examining molecular genetic structure in different populations, molecular anthropologists can figure out how closely related those populations are. Certain similarities in genetic makeup let to determine whether or not different groups of people belong to the same haplogroup, and thus if they share a common geographical origin. This is significant because it allows anthropologists to trace patterns of migration and settlement, which gives helpful insight as to how contemporary populations have formed and progressed over time.

Y-chromosome DNA documents the paternal lineage and becoming a useful tool for tracing human evolution through male lineages. Since the Y-chromosome is passed down from father to son without any recombination, can provide unique insights into the human past. It's long no recombining segment carries the most informative stable haplotypes in the genome, whereas its permanent location in the male genome links these to male specific history.

The Iranian gene pool at different times has been an important source of the Near Eastern and Eurasian Y-chromosome variability as well as a recipient of variation entered with different migratory events (Grugni et al., 2012). Y-chromosomal studies of the modern Iranian populations are indicative of the past settlements and migrations in the Middle East overall, shaping its contemporary patrilineal genetic landscape (Quintana-Murci et al., 2001). The complexity of the Iranian male gene pool is described by previous studies where some of the Iranian groups fall within the Near East and South Asian clusters. Different factors could have contributed to the observed Iranian population heterogeneity, in particular, the presence of important geographic barriers such as the Zagros and Alborz Mountain ranges and the two arid areas, the Dasht-e Kavir and the Dash-e Lut deserts.

∗Corresponding author E-mail:
Bah_ard@yahoo.com

Both types of barriers, running from North-West to South-East, have limited gene flow from neighbouring regions and free movements of internal peoples, starting from the first peopling of this area (Grugni et al., 2012; Nasidze et al., 2008; Yunusbayev et al., 2011; Derenko et al., 2013). Among various geographic features of the territory, Northwest of Iran is a unique and strategic geography due to the proximity to the Fertile Crescent, Mesopotamia, ancient Silk Road passages and its conductive positions of Neolithic agricultural diffusion.

Iranian Azeris are an indigenous population in the Northwest of Iran. The heterogeneous paternal genetic structure in this population also debates regarding the ethnic origins of the Azeris in the modern Iran, requires a reasonable comparative genetic study of Iranian Azeri and other ethnic groups of Iran as well as neighbouring population around the country. Furthermore the current analysis for this paper provides increased resolution based on a larger sample numbers, a collection on deep area and native peoples, additional control populations, higher levels of haplogroup resolution to trace the genetic affinity between the Iranian Azeris and studied populations.

Materials and Methods

The Y chromosome consortium has established a system of defining Y-DNA haplogroups by letters A through to T, with further subdivisions using numbers and lower case letters. Each of these haplogroups originated to certain geography and population and due to human community events such as migrations, nowadays haplogroups are frequent in different geography and ethnic groups. In this method, by finding out frequency of each haplogroup in the population, it is possible to discuss its origin and genetic relation with neighbors.

A total of 297 samples was collected from unrelated healthy male Azeris from three provinces (17 cities) of northwestern Iran (113 samples from the East Azerbaijan province, 140 from the Ardebil province and 44 samples from the West Azerbaijan province). Genomic DNA was extracted from whole blood by using the QIAamp DNA Mini Kit (Qiagene, Hilden, Germany).

UEP and STR genotyping were performed using 48 SNPs based on the human NRY tree published by the Y - chromosome consortium (ISOGG 2011) by PCR -RFLP analysis, Taqman assay (Applied Biosystems, Foster City, CA, USA) or direct sequencing. A set of 48 relevant bi-allelic markers that represent Near and Middle Eastern populations was selected and done base on published conditions (Hammer et al., 2001). Description of main and new Y-chromosome binary markers used in this study are in the Table 1.

A hierarchical strategy for genotyping of the NRY-UEPs was followed using deep-rooting markers of the NRY phylogeny. All samples were genotyped using markers M74, M89 and M9 to define the superclades P, F and K. Then, each sample was systematically genotyped according to the different clades within F, K or P for its final haplogroup designation. Specification for most markers was reported on ISOGG 2011 (http://www.isogg.org/tree).

Table 2. Characteristics of the studied population for Y chromosome dataset.

Region (Population)		COD	Size	Ref
IRAN	Iran- Assyrian	IR-ASY	48	11
	Iran-Persian	IR-PER	160	
	Iran-Gilak	IR-G	64	
	Iran-Turkmen	IR-TU	68	
	Iran-Hormozgan	IR-HBA	143	
	Iran-Qeshmi	IR-HQ	49	
	Iran-Khuzestan(Arab)	IR-ARB	57	
	Iran- Kurd	IR-KUR	59	
	Iran-Lur	IR-LU	50	
	Iran-Mazandarani	IR-MAZ	72	
	Iran-Baluch	IR-BAL	24	
	Iran-Zoroastrian	IR-ZO	47	
	Iranian Azeries	**IR-AZ**	**297**	*
Afghanistan	Afghanistan-Total	AF	190	14
	Afghanistan- Hazara	AF-HA	60	13
	Afghanistan-Pashtun	AF-PA	49	
	Afghanistan-Tajik	AF-TAJ	56	
	Afghanistan-Uzbek	AF-UZ	17	
Iraq	Iraq (Marsh Arab)	IQ-MAR	143	1
	Iraq/Baghdad	IQ-BA	154	
	Pakistan	PAK	176	26
	Armenia-Syunik	ARM-SY	105	**
	Turky Total	TUR	523	5

* This study
** Not published data

A subset of 90 samples belonging to Haplogroups R1b, J1, G and C was also typed for short tandem repeats of Y-chromosome (Y-STRs) using DYS388 (Kayser et al., 1997) and DYS461 (White et al., 1999) as well as 17 STR markers including DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS439, DYS385a, DYS385b, DYS437, DYS438, DYS448, DYS456, DYS458, DYS635, Y_GATA_H4 by Yfiler Kit (Applied Bios-AmpFlSTR, Life technologies, California, USA) based on manufacturer's recommendations. The results were analyzed using the ABI PRISM program Gene Mapper 4.0 (Applied Biosystems).

Same population data from 2314 samples of neighbouring area (Table. 2) was used as a dataset. Genetic Fst distances and gene diversity indices were estimated by Arlequin version 3.5 (Excoffier et al., 2010). Tests for significant population differentiation were carried out using the exact test for population differentiation (Raymond et al., 1995). Similarity matrices based on Fst genetic distances were used to perform principal coordinates analysis (PCO) by GenStat version 14.2. PHYLIP version 3.6 (Felsenstein, 2004) was used to reconstruct neighbor-joining (NJ) tree. Y-STR haplotypes were used to compare populations in R1b, J1e, G and C haplogroups. Primer sequences and concentrations of STR markers presented in Table.3.

Table 3. Primer sequences and concentrations for STR markers genotyping kit.

Primer name	Primer sequence	Dye label	Final concentration (µM)
DYS19-L	CTA CTG AGT TTC TGT TAT AGT	TET	0.236
DYS19-R	ATG GCA TGT AGT GAG GAC A		0.236
DYS388-L	GTG AGT TAG CCG TTT AGC GA	TET	0.318
DYS388-R	CAG ATC GCA ACC ACT GCG		0.318
DYS390-L	TAT ATT TTA CAC ATT TTT GGG CC		0.127
DYS390-R	TGA CAG TAA AAT GAA CAC ATT GC	FAM	0.127
DYS391-L-N[a]	CTA TTC ATT CAA TCA TAC ACC CAT AT	FAM	0.384
DYS391-R-N[a]	ACA TAG CCA AAT ATC TCC TGG G		0.384
DYS392-L-N[a]	AAA AGC CAA GAA GGA AAA CAA A		0.155
DYS392-R-N[a]	CAG TCA AAG TGG AAA GTA GTC TGG	HEX	0.155
DYS393-L	GTG GTC TTC TAC TTG TGT CAA TAC		0.180
DYS393-R	AAC TCA AGT CCA AAA AAT GAG G	HEX	0.088

Results and discussions

We observed an almost similar distribution of the main Y-haplogroups (J2, R1b, R1a and G) in our studied ethnic group and neighbouring populations which is in agreement with previous findings (Grugni et al., 2012; Çinnioğlu et al., 2004; Rootsi et al., 2012) on a westward diffusion of J2-M410*, J2-PAGE55*, J2- M530, G-M201* and R1b-M269* haplogroups and pre-agricultural expansions from the Iranian plateau toward Europe via Caucasus and Turkey.

In this regard all Iranian ethnic groups, including Azeris have shared J2, R1a and G haplogroups with highest frequencies (Supplementary Table 2) while the most frequent haplogroups were J2 and R1in Afghanistan and Pakistan; J2, E1b1 and J1e in Iraq; and J2, G and R1b in Armenia and Turkey.

Distributions of Y-haplogroups

J2-M172 was the most prevalent modal haplogroup in our studied ethnic group. This haplogroup was also reported as the most common haplogroup in the Caucasus, the Fertile Crescent, Anatolia, the Balkans, Italy, the Mediterranean littoral and the Iranian plateau (Semino et al., 2004). The concordance of Iranian Azeris with neighboring populations in this haplogroup frequency, considering the origin of haplogroup J2 from the Middle East (more than 30 KYA) (Nasidze et al., 2008; Di Cristofaro et al., 2013) might suggest geographic distribution of J2-M172 in this part of the world. This issue also agrees and has strong correlation with the diffusion of agriculture from northern Mesopotamia also supported and well documented in the Neolithic archaeological record (Cauvin, 2000; Simone et al., 2013).

The paragroup J2a-PAGE55* was the most frequent of J2 lineage in Iranian Azeris. This paragroup was estimated to be distributed 10.4 KYA in Northwest of Iran, 14.5 KYA in South of Iran, Hormozgan, and 15.5 KYA in the center of Iran (Grugni et al., 2012; Kushniarevich et al., 2013). It is indicative of the distribution of this haplogroup in the Northwest of Iran after other parts of the country and it represents the signature of ancient migratory events in this area which might consider as westward diffusion of this haplogroup and pre-agricultural expansions from the Iranian plateau.

Inverse scattering of R1a and R1b haplogroups has been observed in all studied populations except Iranian Azeris. R1a was more frequent than R1b in Iran, Afghanistan and Pakistan while R1b was more frequent than R1a in Armenia, Turkey and Iraq. South Asian component, R1a, and Asian lineage, R1b, are distributed equally in Iranian Azeris. It is indicative of the merit territory in the Northwest of Iran to transfer and carry important different ancient migratory events such as the recolonizations of Indo-European nomads in the North of Iran, which possibly, linking the spread of R1a to the movement of Kurgan people from North of the Caspian Sea and farmers migration from Near East to Europe during the Paleolithic and the Last Glacial Maximum or in the dispersal process (Karafet et al., 2008; Sikora et al., 2013; Underhill et al., 2014) also varying degrees of demic diffusion and cultural diffusion of R1b

lineage. In aggregate, it emphasizes the conspicuous role of a fertile region in the North of Iran as a gateway for gene flow, of different haplogroups, through the geographical barriers in the West and East of Iran.

The issue also is in aggregation of autochthonous Middle Eastern haplogroup J1-M267 branches, J1e (Page08) and J1-M267*, that display opposite distribution in almost all studied populations except Iranian Azeris.

The haplogroup J1e (Page08), reported likely originated in the border between southeastern Turkey and North parts of Iraq (Grugni et al., 2012; Al-Zahery et al., 2011; Chiaroni et al., 2009), underwent an important Neolithic expansion in the southern countries of the Middle East and represents one of the principal haplogroups in the modern populations of the Arabian Peninsula and North Africa. Nonuniform distribution of this lineage in different geographical locations of Iran, particularly in both sides of the Zagros Mountains, indicates a possible barrier role of geographical boundaries as Zagros Mountains which hamper the flow of this linage to the other sides of Iran.

Haplogroup J1-M267* shows high variance in the Middle Eastern region including Eastern Turkey, North-West Iraq (Rootsi et al., 2012; Semino et al., 2004; Haber et al., 2013) and North-West Iran, where probably originated and then migrated westwards to the Balkans and the Italian Peninsula and southwards as far as in Saudi Arabia and Ethiopia (Grugni et al., 2012). The proportion of these two sub-lineages is highly variable in Iran. J1-M267* is almost restricted among northwestern Iranian ethnic groups and J1-Page08 is mainly observed in populations living below the Dasht-e Kavir and Dasht-e Lut deserts. It reaches a frequency of 32% among Arabs of Khuzestan on the southern border of Iraq (Wells et al., 2001; Di Giacomo et al., 2004), while in Iranian Azeris both lineages are in close portion which is suggested the origin of these lineages.

Despite the general similarity according to the dispersal of major haplogroups in studied populations, some minor haplogroups in Iranian Azeris seems entered from the eastern or northern neighbors: Central Asian components (Q, C and O) which are frequent in Eastern countries as Afghanistan and Pakistan, also European lineage, I, which is more frequent in Armenia and Turkey, comparing to Iranian Azeris, and haplogroup J1e-Page55 that frequently reported in western neighbors of Iran, as Iraq, in addition south Asian component (H, R2, L and N2) that is frequent in Afghanistan and Pakistan.

Detected gene diversity values, *h*, in most of the examined population, lies within the similar high range (0.9-0.8) except the low diversity exhibited by the Iranian Turkmen, Iraqi Marsh and Afghani Pashto (**Figure1**).

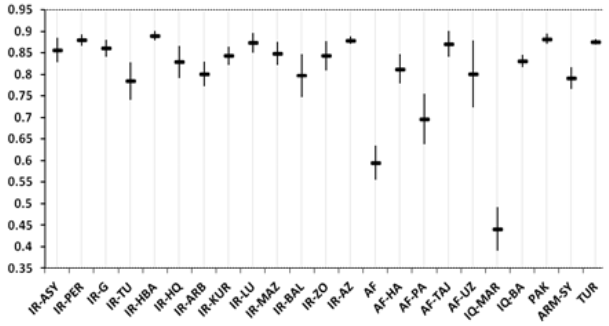

Figure 1. Genetic diversity (*h*) values, based on the Y-chromosome haplogroup frequency, with standard deviations in the studied populations.
X axis: estimated genetic diversity by Arlequin version 3.5 .
Y axis: studied Populations. Abbreviations used are described in the Table 2.

The similar range of diversity among most Iranian ethnic groups is in agreement with previously observed studies (Wells et al., 2001; Semino et al., 2004; Quintana-Murci et al., 2004; Rafiee et al., 2009) and is in concordance with Population Pairwise differences in *h* values (Supplementary Table 3).

No significant difference has been observed among Iranian ethnic groups in Pairwise differences in *h* values, except Turkmens that significantly differ from Azeris, Persians and Iran-Hormozgan. In this regard, Iranian Azeris showed a high level of gene diversity (0.8796) which was significantly different in comparison with the Arab and Turkmen ethnic groups of Iran, Afghan Pashto, both Iraqi sub-populations and Armenian Syunic ($P<0.05$). The high gene diversity values, *h*, detected throughout Iran as a multi-ethnic and multi-linguistic region points to its central role as a strategic multidirectional gateway intersecting three continents and four major linguistic families (Quintana-Murci et al., 2001). This pattern is particularly seen in the west part of the Zagros Mountains, inhabited by Iranian Azeris, Lurs, Arabs and Kurds. It is consistent with an early settlement of the fertile region by modern humans followed by subsequent migration routes across the world. It also possibly explains the peculiarity of the Northwest of Iran as a corridor for ancient human migration. The lowest level of genetic diversity is encountered in the Iranian Turkmen, Iraqi Marsh and Afghan Pashto, which reflects their long centuries of reproductive isolation due to their language, religion and other cultural features.

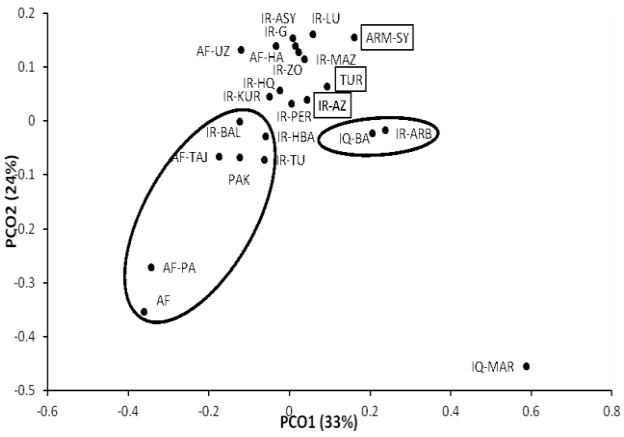

Figure 2. Principal coordinates (PCO) plot based on the F_{ST} values of studied populations using Y chromosome genotyping data. Numbers in brackets show the percentage of total variation explained by each axis. Populations close together in the circles have less genetic distance. Abbreviations used are described in the Table 2.

Genetic distance analysis

Using Y-chromosomal haplogroup frequencies we have assessed the genetic relationship between all populations under consideration by calculating the conventional F_{ST} genetic distances. The results are displayed in **Supplementary Table 4**, showing that based on the F_{ST} genetic distances Azeris, Lurs and Parsees of Iran are closer to each other and significantly ($p< 0.05$) differ from other ethnic groups of Iran and other studied populations based on the pairwise *P* value results. Interestingly, the Iranian populations are significantly far from all other neighbouring populations.

In order to depict the pattern of genetic affinities between Iranian Azeris and comparative data sets in a two-dimensional space, PCO analysis has conducted based on F_{ST} genetic distances (**Figure 2**). As shown, Iranian Arabs are clustered to other Arab populations (IQ-Mar and IQ-BA) which geographically are, also close to the west borders of Iran. Iranian Balochis and Hormozgan population are clustered to Eastern neighbors like Afghans and Pakistani population. In agreement to PCO plot, Pairwise F_{ST} values showed similarity between the Iranian Baloch and Afghan-TAJ. Iranian Azeris is located near the northwestern neighbors like Turks and Armenians and other Iranian ethnic groups in the north (Gilak and Mazandarani) as well as Lurs in the northwest of Iran. Paternal heterogeneity of Iranians also could infer geographical boundaries such as Zagros Mountains, Dasht-e-kavir and Dasht-e-Lut serve as important geographic barriers to gene flow from/to neighbouring regions.

To visualize the genetic relationship among Iranian Azeris and other populations of the area NJ tree was constructed based on FST genetic **(Figure 3).** These results, also demonstrates the correlation between the geographic proximity and genetic structures of these populations. As shown in the tree, all Iranian ethnic groups are located in a separate branch. Iranian Arabs show a common ancestor with Arab populations of Iraq, Iranian Baloch, Turkmen and the people of Hormozgan are located on the same branch of eastern neighbors, Afghanistan and Pakistan, and Iranian Azeris are located close to northwest neighbors, Turkey and Armenian populations.

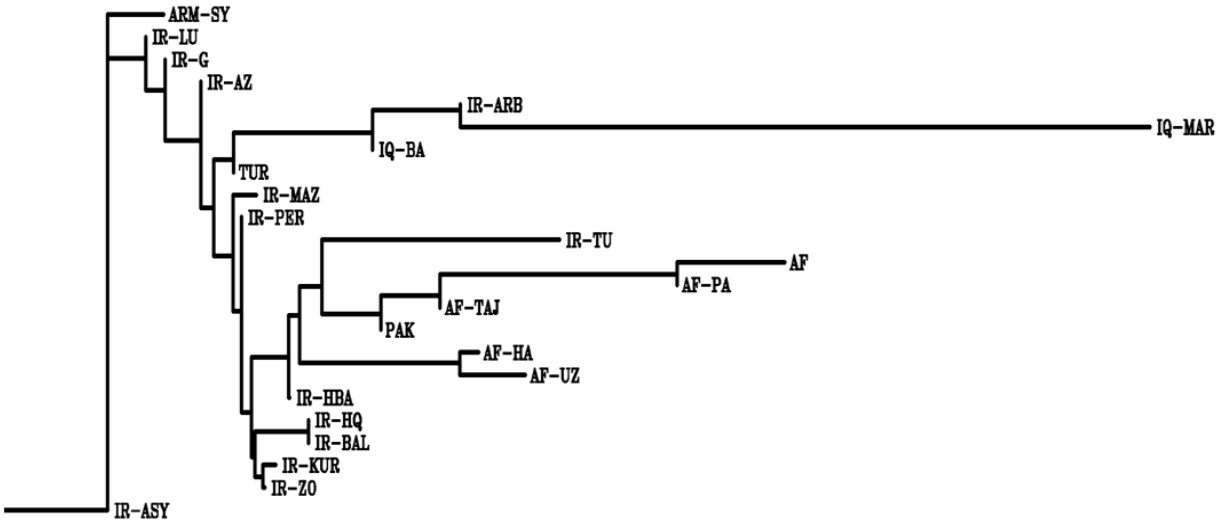

Figure 3. Rooted Neighbor-Joining dendrogram of the studied populations based on FST genetic distances of Y chromosome markers. Clustering of the distance between each pair of populations and root them according to their genetic distance. Populations on the same root have less genetic distance. Abbreviations used are described in the Table 2.

Microsatellite haplotype analysis

STR markers provide another level of differentiation of the populations. Following the aim of the study to find out the genetic affinity between the Iranian Azeris and other populations, in four major haplogroups (G-M201, J1, R1b-M343 and C-M130) results were analyzed at the haplotype level, as defined by STRs; DYS19, DYS389a, DYS389b, DYS390, DYS391, DYS392, DYS393 and DYS439. Only those haplotypes within the same haplogroup (i.e. Lineages) were analyzed together (Supplementary Table 5). For each haplogroup, haplotypes in the different population were compared with corresponding haplotype considering modal and shared haplotypes. Therefore, we present here the detailed table of all observed SNP+MS haplotypes and their frequencies in five populations (Supplementary Table 5) also encountered modal haplotypes for each population presented in Table 4.

Table 4. Frequently encountered Microsatellite (STR) haplotypes in the studied populations. STR markers used in these lotypes are DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS439 respectively from left to right.

Haplogroup	**Microsatellite haplotype**	**Ir-Az** (*n=88*)	**Iranian** (*n=77*)	**Afghan** (*n=44*)	**Turkey** (n=195)	**Arm-Syu** (*n=68*)
R1b-M343	14 16 13 24 11 13 12 12	-	-	-	**0.036**	-
R1b-M343	14 13 15 24 11 14 12 13	**0.045**	-	-	-	-
R1b-M343	14 13 16 24 11 13 12 11	-	0.039	-	-	0.063
J1e-Pag09	14 13 16 23 10 11 12 11	**0.045**	0.026	-	-	0.016
J1e-Pag08	14 13 16 23 11 11 13 11	-	-	-	-	**0.078**
J1e-Pag08	14 13 17 23 10 11 12 11	-	**0.052**	-	-	0.016
G-M201	15 12 16 22 10 11 14 12	**0.045**	0.013	-	-	-
C-M130	16 13 16 25 10 11 13 10	-	-	**0.182**	-	-

The microsatellites together with mentioned SNP haplogroups defined 367 haplotypes in total; 69 haplotypes in Iranian Azeris, 64 in Iranians, 26 in Afghans, 143 in Turks and 42 in Armenians, which totally 23 haplotypes shared between populations (Supplementary Table 5).

The modal haplotypes in Iranian Azeris interestingly belong to haplogroups G-M201, R1b-M343 and J1e-Pag08 (equally 0.045) which for J1e, modal haplotype overlapped (also shared) by Iranian and Armenians and for G-M201, modal haplotype overlapped by Iranians. These results reveal a heterogeneous paternal genetic structure in Iranian Azeris. The modal haplotype of Afghanistan belongs to haplogroup C-M130 which originates from central Asia, did not observe in other populations.

Haplogroup J1e in haplotype level is modal and frequently observed in Iranian Azeris, Iranians and Armenian Syunic population. It is generally in agreement with previous reports that the haplogroup was dispersed by the westward movement of the people from the Middle East (Çinnioğlu et al., 2004; Renfrew, 1996; Simone et al., 2013; Behar et al., 2013) and might inference origin of J1e nearby eastern Anatolia or sough Caucasia.

Haplogroup R1b in haplotype level is modal in the Iranian Azeris and Turkey also frequently observed in the Iranians and Armenian Syunic population. Therefore, this issue provides a genetic signature of the Eurasia paternal gene pool, and bear witness to the expansion of this lineage across the continent after the Last Glacial Maximum and agreed previous reports that the haplogroup was originated in Asia and lies in Eurasia, most likely in Western Asia (Sikora et al., 2013).

Conclusion

In this work, we have shown the major mechanisms responsible for shaping the genetic structure of the modern Iranian Azeri population using Y-chromosomal markers which are sensitive tools in population genetics studies. Specifically, we have unmasked the major factors that have assigned strategic feature for Northwest of Iran.

The heterogeneous paternal genetic structure for the Iranian Azeris was statistically supported by different tests, particularly at the STR marker haplotype level. The Central Asian haplogroups have a notable contribution (6.4%) to the Iranian Azeri paternal gene pool. Hence the geographic location of northwest of Iran may have facilitated Mongol and other Turkic-speaking tribes from the Central Asian steppes in the thirteenth century CE therewith elite dominance model for Turkic

language dispersal have shaped the population structure of Northwest of Iran.

The high level of genetic diversity detected in Iranian Azeris is an evidence of the peculiarity of the region as a constituent part of the ancient Silk Road, as well as a settlement area for pre-Islamic Iranian people of the Central Asian origin.

In summary, our collection of samples and dataset, that cover the full extent of ethnic groups of Iran and neighboring population, shows that most Iranians proportionally contribute the majority of their genome with together and share varying minor proportion with their geographic neighbors, suggesting and emphasizing the importance of the Iranian Plateau as a source and recipient of gene flow between culturally and genetically distinct populations. Hence minor differences in genetic structure of the Iranian ethnic groups can be explained by taking into account their geographical locations in the territory.

References

1. Al-Zahery N., Semino O., Benuzzi G., Magri C., Passarino G., Torroni A. and Santachiara- Benerecetti AS. (2011)Y-chromosome and mtDNA polymorphisms in Iraq, a crossroad of the early human dispersal and of post-Neolithic migrations. Mol Phylogenet Evol 28:458-472.
2. Behar D., Metspalu M., Yael B., Naama KM., Bayazit Y., Ariella G., Shay T., Sahakyan H and Bahmanimehr A.(2013) No Evidence from Genome-Wide Data of a Khazar Origin for the Ashkenazi Jews. Human Biology Open Access Pre-Prints Paper 41. 2013; http://digitalcommons.wayne.edu/humbiol_preprints/41.
3. Cauvin J. (2000) The Origins of Agriculture in the Near East: a Symbolic Interpretation (English edition). Cambridge University Press. Cambridge, 125pp.
4. Chiaroni J., King R.J., Myres N.M., Henn B.M., Ducourneau A., Mitchell M.J., Boetsch G., Sheikha I., Lin A.A., Nik-Ahd M., Ahmad J., Lattanzi F., Herrera R.J., Ibrahim M.E., Brody A., Semino O., Kivisild T. and Underhill P.A. (2009)The emergence of Y-chromosome haplogroup J1e among Arabic-speaking populations European. Journal of Human Genetics 18: 348–353.
5. Çinnioğlu C., King R., Kivisild T., Kalfoglu E., Atasoy S., Cavalleri G.L. and Lillie A.S. (2004) Excavating Y-chromosome haplotype strata in Anatolia. Hum Genet 114:127-148.
6. Derenko M., Malyarchuk B., Bahmanimehr A., Denisova G and Perkova M. (2013) Complete Mitochondrial DNA Diversity in Iranians. PLoS ONE 8(11): e80673. doi:10.1371/journal.pone.0080673.
7. Di Cristofaro J., Pennarun E., Mazie`res S., Myres NM and Lin AA. (2013) Afghan Hindu Kush: Where Eurasian Sub-Continent Gene Flows Converge. PLoS ONE 8(10): e76748. doi:10.1371/journal.pone.0076748.
8. Di Giacomo F., Luca L. O., Popa N., Akar N., Anagnou J. and Banyko R. (2004)Y-chromosomal haplogroup J as a signature of the post-neolithic colonization of Europe. Hum Genet 115:357–371.
9. Excoffier L. and Lischer H.E. (2010) Arlequin suite Ver 3.5: a new series of Programs to perform population genetics analyses under Linux and Windows. Mol Ecol Resour 10:564-567.
10. Felsenstein J. (2004) PHYLIP (Phylogeny Inference Package) version 3.6, Distributed by the author, Department of Genome Sciences, University of Washington, Seattle.
11. Grugni V., Battaglia V., Hooshiar Kashani B., Parolo S., Al-Zahery N., Achilli A., Olivieri A., Gandini F., Houshmand M., Sanati M., Torroni A. and Semino O. (2012) Ancient Migratory Events in the Middle East: New Clues from the Y-Chromosome Variation of Modern Iranians. PLoS ONE7:7,:e41252.
12. Haber M., Gauguier D., Youhanna S., Patterson N and Moorjani P. (2013) Genome-Wide Diversity in the Levant Reveals Recent Structuring by Culture. PLoS Genet. 9:2; e1003316. doi:10.1371/journal.pgen.1003316.
13. Haber M., Platt D.E., Ashrafian Bonab M., Youhanna S.C and Soria-Hernanz D.F. (2012) Afghanistan's Ethnic Groups Share a Y-Chromosomal Heritage Structured by Historical Events. PLoS ONE 7:3. e34288. doi:10.1371/journal.pone.0034288.
14. Harlette L., Tenzin G., Regueiro M., Chennakrishnaiah S., Bukhari A., Underhill P.A. Garcia-Bertrand R.L. and Herrera R.J. (2012) Afghanistan from a Y-chromosome perspective. European Journal of Human Genetics 1–8. doi:10.1038/ejhg.2012.59.
15. Karafet T.M., Mendez F.L., Meilerman M.B., Underhill P.A., Zegura S.L. and

Hammer M.F. (2008) New binary polymorphisms reshape and increase resolution of the human Y-chromosomal haplogroup tree. Genome Res 18:830-838.

16. Kayser M., Caglia A. and Corach D. (1997) Evaluation of Y-chromosomal STRs: a multicenter study. Int J Legal Med 110:141-149.
17. Kushniarevich A., Sivitskaya L., Danilenko N., Novogrodskii T and Tsybovsky I. (2013) Uniparental Genetic Heritage of Belarusians: Encounter of Rare Middle Eastern Matrilineages with a Central European Mitochondrial DNA Pool. PLoS ONE. 8(6): e66499. doi:10.1371/journal.pone.0066499.
18. Nasidze I., Quinque D., Rahmani M., Alemohamad S. and Stoneking M. (2008) Close genetic relationship between Semitic-speaking and Indo-European-speaking groups in Iran. Ann Hum Genet 72:241-252.
19. Quintana-Murci L., Chaix R., Wells S., Behar D., Sayar H., Scozzari R., Rengo C., Al-Zahery N., Semino O. and Santachiara-Benerecetti A. (2004) Where West meets East: The complex mtDNA landscape of the Southwest and Central Asian corridor. Am J Hum Genet 74:827-845.
20. Quintana-Murci L., Krausz C., Zerjal T., Sayar S.H. and Hammer M.F. (2001) Y-chromosome lineages trace diffusion of people and languages in southwestern Asia. Am J Hum Genet 68:537-42.
21. Rafiee M.R., Sokhansanj A., Naghizadeh M.A. and Farazmand A. (2009) Analysis of Y-Chromosomal Short Tandem Repeat (STR) Polymorphism in an Iranian Sadat Population. Russian Journal of Genetics 45:969-973.
22. Raymond M. and Rousset F. (1995) An exact test for population differentiation. Evolution 49:1280-1283.
23. Renfrew C. (1996) Languages families and the spread of farming. In: Harris DR (ed) The origins and spread of agriculture and pastoralism in Eurasia. Smithsonian Institution Press. Washington DC, USA, 217-224pp.
24. Rootsi S., Myres N.M., ALin A., Jarve M., King R.J., Kutuev I., Cabrera V.M., Khusnutdinova E.K., Varendi K., Sahakyan H., Behar D.M., Khusainova R., Balanovsky O., Balanovska E., Rudan P., Yepiskoposyan L., Bahmanimehr A., Farjadian S. and Villems R. (2012) Distinguishing the co-ancestries of haplogroup G Y-chromosomes in the populations of Europe and the Caucasus. European Journal of Human Genetics 1:8, doi:10.1038/ejhg.2012.86.
25. Semino O., Magri C., Benuzzi G., Lin A.A., Al-Zahery N., Battaglia V., Maccioni L., Triantaphyllidis C., Shen P., Oefner P.J., Zhivotovsky L.A., King R., Torroni A., Cavalli-Sforza L.L., Underhill P.A. and Santachiara-Benerecetti A.S. (2004) Origin, diffusion, and differentiation of Y-chromosome haplogroups E and J: inferences on the neolithization of Europe and later migratory events in the Mediterranean area. Am J Hum Genet 74:5, 1023-34.
26. Sengupta S., Zhivotovsky L.A., King R., Mehdi S. Q., Edmonds C.A., Rani U., Thakur C.M., Cavalli-Sforza L. and Underhill P.A. (2006) Polarity and Temporality of High-Resolution Y-Chromosome Distributions in India Identify Both Indigenous and Exogenous Expansions and Reveal Minor Genetic Influence of Central Asian Pastoralists. Am. J. Hum. Genet 78:202–221.
27. Sikora M. J., Colonna V., Xue Y. and Tyler-Smith C. (2013) Modeling the contrasting Neolithic male lineage expansions in Europe and Africa. Investigative Genetics 4:25. doi:10.1186/2041-2223-4-25.
28. Simone R., Mohsen Z. and Nicholas J. C. (2013) Emergence of Agriculture in the Foothills of the Zagros Mountains of Iran. Science. 341: 65; doi:10.1126/science.1236743.
29. Simone R., Zeidi M. and Conard N.J. (2013) Emergence of Agriculture in the Foothills of the Zagros Mountains of Iran. Science 341:65, 64-67. doi: 10.1126/science.1236743.
30. Underhill P.A., Poznik G.D., Rootsi S., Jarve M., Lin A.A., Wang J., Sahakyan H., Behar D.M., Kushniarevich A., Sarac J., Chaubey G., Grugni V., Semino O., Yepiskoposyan L., Bahmanimehr A., Farjadian S., Balanovsky O., Chiaroni J. and Villems R.(2014)The phylogenetic and geographic structure of Y-chromosome haplogroup R1a. European Journal of Human Genetics. 1:8, 1018-4813/14
31. Wells R.S., Yuldasheva N., Ruzibakiev R., Underhill P.A. and Evseeva I. (2001) The Eurasian heartland: a continental

perspective on Y-chromosome diversity. Proc Natl Acad Sci USA 98:10244-9.

32. White P.S., Tatum O.L., Deaven L.L. and Longmire J.L. (1999) New male-specific microsatellite markers from the human Y-chromosome. Genomics 57:433-437.
33. Yunusbayev B., Metspalu M., Jarve M., Kutuev I., Rootsi S., Metspalu E., Behar D.M., Varendi K., Sahakyan H., Khusainova R., Yeppiskoposyan L., Khusnutdinova E.K., Underhill P.A., Kivisild T. and Villems R. (2011)The Caucasus as an asymmetric semi permeable barrier to ancient human migrations. Mol Biol Evol. 29:1,359–365.

Table 1. Description of some main and new Y-chromosome binary markers used in this study.

Common Name Marker	YCC nomenclature Haplogroup	Nucleotide change	Amplicon size (bp) reference sequence	Polymorphism position from 5' end of + strand	Restriction enzyme	Primer Forward 5'-3'	Primer reverse 5'-3'
M130	C*	C to T	205	41	BseL1	CTGCCCAGGGGAAAGGGCAT	CCACAAGGGGGAAAAAACAC
M78	E1b1b1a	C to T	301	197	AciI	GGGGTAACATTGGACATTCATTGCA	ATAGTGTTCCTTCACCTTTCCTT
M285	G1*	G to C	287	70	HphI	TTATCCTGAGCCGTTGTCCCTG	TGTAGAGACACGGTTGTACCCT
M485	G2a3*	C to T	312	150	Mva I	CTCATTTCCTCACATGTATGC	TTTAGGAATTACTATGTAGCGTC
M547	G2a3*	T to G	423	284	HpaII	AGAGATGGGTTTTCACCGTG	GCATAAATGTCAAGCCCACTAG
M461	G2a3a	C to T	329	114	BseYI	GCAGAAATGAAAGATGGCTG	TGAATCACACTACTCCCACG
M527	G2a3b1a1	C to T	327	128	Af1II	GTTTCATGGGAATAAACACTGG	AGTATCAAAGCACATGTGTTGC
M426	G2a3b1b	T to G	337	221	AlwI	ACTTAAACCTAAGTCATTTTGGGTG	GATCATCGGAAGTGACAGCC
M69	H	T to C	257	222	HpaII	AGCTTCAGGAGGCTGTTTACAC	AAAATATATTTCAGCAAGACAAAGG
M253	I1	C to T	400	283	Hinc II	GCAACAATGAGGGTTTTTTTG	CAGCTCCACCTCTATGCAGTTT
M267	J1*	T to G	287	148	Mva I	TTATCCTGAGCCGTTGTCCCTG	TGTAGAGACACGGTTGTACCCT
Page8	J1e	T to C	306	189	HpyF3I	ACGTCACCCATCTCAACATC	AAAGAATGTCTCCCCATGAGG
M67	J2a2*	A to T	409	377	AluI	GTGATGACAAACTCCCCTGC	GTCTTTTCACTTGTTCGTGGAC
M92	J2a2a	T to C	470	340	Eco1051	TTCAGAAACTGGTTTTGTGTCC	TTCAGAAACTGGTTTTGTGTCC
M242	Q	C toT	337	180	HphI	AACTCTTGATAAACCGTGCTG	TCCAATCTCAATTCATGCCTC
Z282	R1a1a1a*	T to C	297	155	AluI	GTTCTACAGGTTACAGGTTAGC	GGGAAACAAAAACATTCC
Z284	R1a1a1a1	C to G	275	176	Bg1II	GAGAATTTCAAAAATCATCC	GGGAAACAAAAACATTCC
M458	R1a1a1b1a1	A to G	380	87	BsrI	AGAAGAGATTTCTAGCCAGAGT	GGGGTAGAAAATTATTGGTC
Z280	R1a1a1b1a2	C to T	120	64	AlwI	GCATAATTACTGCTGTCATCTTCC	CAAAGGTCTTTACTTGTGCAATATC
M558	R1a1a1c*	T to C	281	211	AvaII	TGTTGGCTGGCCTCTCTC	GAACAAGGCAGTTGTAGGATAG
M582	R1a1a1c*	T to G	273	111	Tsp509I	GAGGCTGCAGTGAGCTATGAC	GTCACCTGCTTGGTAAAGATGAC
Z93	R1a1a2*	G to A	338	172	AluI	AACAAAGCATCATCAAAGGC	CATGATTCGTTATGACCTGC
Z95	R1a1b2a*	C to T	429	153	BsrI	TCTTTTCTGACTGGCCAGG	GGCTTATCTTTCTGTTTCCTGAAG
Z2125	R1a1b2a2*	C to T	895	284	HpyCH4 III	CCAAACCCCAGTGCCAGC	CCTAAGGCCAGGGAAGGCTC
M204	R1a1b2a2a	T to G	486	234	SfcI	AAGGGGCGAAGTATTCCAGAG	TGAAGAGGAGTCTGTTAGCCTG
M434	R1a1b2a2b	G to A	320	213	BseYI	CCAAAATTAGTGGGGAATAGT	GATCACCCAGGGTCTGGAGTT
M560	R1a1b2a3*	G to T	305	151	HphI	TGTAGATGATGGGTTAATGGGTG	GCACATAATATGTTTGAGAAGGC
M780	R1a1b2a4*	C to T	386	130	HpaII	GAAGATCCAAAACCTAAGAGAAC	GCTCAATGAGGAAGGCGATC
M70	T	A to C	257	45	SfcI		ATCTTTATTCCCTTTGTCTTGCT

Research Note

PCR-SSCP: A method to fingerprint sequence variability in plant gene pools using the Iranian wild diploid *Triticum*

Fatemeh Nasernakhaei, Mohammad Reza Rahiminejad*, Hojjatollah Saeidi and Manoochehr Tavassoli

Department of Biology, Faculty of Sciences, University of Isfahan, Isfahan, Iran

Received 15 Jan 2014 Accepted 01 March 2014

Abstract

As a case study, the genotypic polymorphism of the Iranian material of diploid *Triticum* was screened based on internal transcribed spacer 2 (ITS2) of the ribosomal DNA (rDNA) using single strand conformation polymorphism (SSCP) analysis. This is a simple and cost effective technique for genotyping and investigating the allelic polymorphism among plant populations. Performing SSCP analysis among 21 accessions, two distinctive migration profiles associated with the two validly recognized diploid *Triticum* species i.e., *T. monococcum* subsp. *aegilopoides* and *T. urartu* were detected. Nucleotide sequencing confirmed the distinction of two types of observed profiles. This study suggested that SSCP is an applicable diagnostic molecular tool for screening the genotypic variability before the costly direct genomic sequencing is begun.

Keywords: diploid, Iran, ITS2, SSCP, *Triticum*

Introduction

Single strand conformation polymorphism (SSCP) is a simple and useful method for detecting the sequence variants and polymorphisms (Hayashi, 1991). Principle of this technique relies on electrophoretic mobility of the single strand DNA molecule on a non-denaturing gel according to its size and conformation (Rodriguez *et al*., 2011). Small nucleotide sequence changes result in alteration of conformation of single strand DNA which in turn alters the electrophoretic mobility and consequently reveals different SSCP patterns (Hayashi, 1991). Size of the fragment, base composition of the sequence, electrophoresis temperature, pore size and cross linking of gel are the effective parameters for polymorphism detection in SSCP technique (Gasser *et al*., 2006).

Since its invention by Orita *et al*. (1989) SSCP approved to be an effective method in different fields of both basic and applied biology (Sunnucks *et al*., 2000; Gasser and Chilton, 2001), briefly its utilization in plant science is summarized in Table 1. In addition, in the laboratories with a minimum of facilities, SSCP can be a suitable alternative for direct analysis of sequence variation.

DNA barcoding is an increasingly popular specific identifying tool with respect to its affordable cost, speed and objectivity (Crautlein *et al*., 2011). ITS2 region can be used as a barcode in plants because of its valuable characteristics, including the availability of conserved regions for designing universal primers, the ease of its amplification, and enough variability to distinguish even closely related species (Yao *et al*., 2010; Chen *et al*., 2010). In order to initiate and set up the utilizing this method (SSCP) in plant taxonomy in our lab, we select *Triticum* diploid species as a case study using ITS2 amplicons.

Materials and methods

Plant materials

Our plant materials include 21 seed accessions collected from the wild (Table 2) which were belonged to precisely identified diploid species of *Triticum* i.e., *T. urartu* Thumanjan ex Gandilyan and *T. monococcum* subsp. *aegilopoides* (Link) Thellung. The accessions were grown in the research filed of the Isfahan University in 2011. All the examined accessions were deposited with at least one specimen in the herbarium of the University of Isfahan.

∗Corresponding author E-mail:
mrr@sci.ui.ac.ir

Table 1. A summarized review of using SSCP in plant biology.

SSCP application in:	Reference
Point mutations detection	To *et al*., 1993
Genome mapping	Fukuoka *et al*., 1994
Population genetic, DNA biogeograghy	Watano *et al*., 1995
Gene mapping, genetic diversity	Bodenes *et al*., 1996
Rare polymorphisms detection	Dumolin-Lapegue *et al*., 1996
Plasmon analysis	Wang *et al*., 1997
Comparative genomic mapping	Plomion *et al*., 1999
Phylogeny	Kita and Ito, 2000
Single nucleotide mutations detection	Martins-Lopes *et al*., 2001
Phylogeography	Meusnier *et al*., 2002
Mutation detection	Sato and Nishio, 2003
Genetic structure of hybrid zones	Watano *et al*., 2004
Genome diversity and gene haplotypes	Salmaso *et al*., 2004
Biological status of complex fern	Ebihara *et al*., 2005
Comparative genomic mapping	Castelblanco and Fregene, 2006
Cytoplasmic DNA characterization of somatic hybrids	Olivares-Fuster *et al*., 2007
Genetic diversity	Kuhn *et al*., 2008
Polymorphism detection	Lu *et al*., 2009
Marker assisted selection	Borchert and Hohe, 2009
Phylogeny	Rousseau-Gueutin *et al*., 2009
Polymorphism detection	Karatas *et al*., 2010
Diversity analysis and varietal identification	Swapna *et al*., 2011
Separation of alleles from polyploid accessions	Rodriguez *et al*., 2011

DNA extraction

DNA was extracted from fresh and young leaves of a single plant, using the CTAB method (Gawel and Jarret, 1991).

PCR amplification

The ITS2 regions were amplified by PCR using primers PITS3 (5'- ACG CCA AAA CAC GCT CCC AAC -3', wheat specific primer) (Blatter *et al*., 2002) and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (White *et al*., 1990). The thermal and $MgCl_2$ concentration gradient were optimized and the final PCR of ITS2 region was carried out in a total volume of 25 µl containing 40–50 ng of DNA template, 200 µM of each dNTPs, 0.4 µM for each of the primers, 2.5 µl 10 × PCR buffer, 3 mM $MgCl_2$ and 1 U *Taq* DNA polymerase. PCR conditions were: 1 cycle of 94ºC for 5 min followed by 30 cycles of 94ºC for 1 min, 52ºC for 1 min and 72ºC for 1 min and finally 1 cycle of 72°C for 10 min. The PCR products were electrophoretically separated in 1.2% agarose gel.

SSCP and sequencing analyses

The SSCP analysis of amplified gene fragments is carried out as described by Rodriguez *et al*. (2011) with some modifications. Briefly, 1–2 µl of PCR products (according to the band intensity on agarose gel) were mixed with 10 µl of a denaturing buffer (95% formamide, 10 mM EDTA, 10 mM NaOH, 1% xylene cyanol and 1% bromophenol blue). After a short spin, mixtures were heated at 100°C for 5 min, chilled on ice for 5 min and then loaded onto a 10% acrylamide: bis acrylamide (39: 1) non-denaturing polyacrylamide gel (25 cm × 20 cm × 1 mm) containing of 5% glycerol. Before sample loading, the gel was pre-run in 1 × TBE for about 1 h at 8°C. Electrophpresis was performed at 200 volts for 17–18 h at 8°C. SSCP bands were visualized by silver staining as described by Sanguinetti *et al*. (1994). In order to corroborate the SSCP results five PCR products from each species were randomly selected and sequenced. The sequence data were aligned manually using BioEdit ver.7 (Hall, 1999). The editing of sequences was checked through ChromasPro ver.1.41 (Technelysium Pty Ltd., Tewantin, Australia). The analysis of sequences was performed using DnaSP ver.5.10 (Liberado and Rozas, 2009).

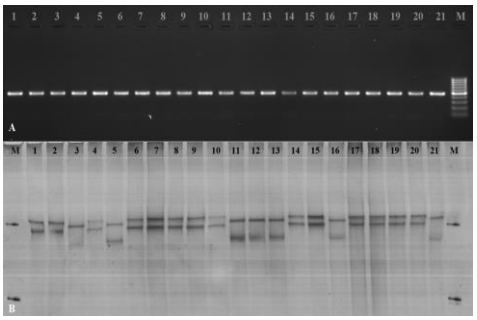

Figure 1. PCR products on agarose gel (A) and SSCP profiles on the non- denaturing acrylamide gel (B) based on the ITS2 region of wild diploid Triticum species. For the number of each line see Table 2. Lane M is 50 bp Ladder.

Table 2. Locality of seed samples of wild diploid *Triticum* species in this study.

No.	Taxon	Locality
1	*T. monococcum*. subsp. *aegilopoides*	Ardabil, 35 km to Givi
2	*T. monococcum*. subsp. *aegilopoides*	Ilam, Gardaneh Reno
3	*T. urartu*	Chaharmahal and Bakhtiari, Cheshmeh Ali
4	*T. monococcum*. subsp. *aegilopoides*	East Azarbaijan, Maragheh
5	*T. urartu*	Chaharmahal and Bakhtiari, Ardal to Dashtak
6	*T. monococcum*. subsp. *aegilopoides*	Isfahan, Bouin to Aligodarz
7	*T. monococcum*. subsp. *aegilopoides*	Kermanshah, Taghi abad
8	*T. monococcum*. subsp. *aegilopoides*	Kurdistan, 5 km to Kamyaran
9	*T. monococcum*. subsp. *aegilopoides*	Kermanshah, Abolvafa
10	*T. monococcum*. subsp. *aegilopoides*	Kermanshah, after Kohdasht to Eslam abad
11	*T. urartu*	Isfahan, Bouin to Aligodarz
12	*T. urartu*	Kermanshah, 10 km to Songhor
13	*T. urartu*	Kohgiluyeh and Boyer-Ahmad, near Yasouj
14	*T. monococcum*. subsp. *aegilopoides*	West Azarbaijan, Piranshahr
15	*T. monococcum*. subsp. *aegilopoides*	Kurdistan, Marivan
16	*T. urartu*	Kurdistan, Marivan
17	*T. monococcum*. subsp. *aegilopoides*	Kurdistan, Sanandaj
18	*T. monococcum*. subsp. *aegilopoides*	Lorestan, 35 km to Khoram abad
19	*T. monococcum*. subsp. *aegilopoides*	Lorestan, Khoram abad to Sefid dasht
20	*T. monococcum*. subsp. *aegilopoides*	Lorestan, Sefid dasht
21	*T. urartu*	Kurdistan, Jenyan

Results

The PCR products (double strand DNA) of the amplified fragments in both Triticum species were aligned for a length of 270 bp (Figure 1- A). The two banded SSCP profiles among the 21 accessions examined revealed two species specific patterns associated with *T. urartu* and *T. monococcum* subsp. *aegilopoides* (Figure 1- B). The patterns were reproducible in repeated experiments. All the five repeated PCR products sequenced in each species were identical and comparing the patterns among the two species revealed two different haplotypes separated on two SNPs (Figure 2).

Discussion

This study showed that prior to a costly direct genomic sequencing SSCP can be a cost effective method when researchers are facing with a large number of accessions. In addition, compared with the other molecular fingerprinting methods it is simple and efficient. While fragments with the same size but different in their sequences migrate to the same position on agarose gel, using the non-denaturing acrylamide, these fragments have different mobility shift and consequently turn in allelic sequence variations (Cotton, 1997) (Figure 1: - A and -B) which can be interpreted easily.

On the basis of the two banded profiles detected it can be concluded that all the accessions studied were homozygote for ITS2. SSCP profiles divided the studied accessions into two groups which were exactly coincidence with our taxonomic species, i.e., *T. monococcum* subsp. *aegilopoides* and *T. urartu*. Therefore ITS2 can be used for species identification. This result is in agreement with Chen *et al*. (2010) and Yao *et al*. (2010).

We found no geographical subdivision or intraspecific variation which can be taken as evidence for the highly conserved nature of ITS2 in these taxa.

Plant species are primarily distinguished by their morphological features; however poor constructed keys or flawed specimens can cause misidentifications or doubtful determinations. In diploid *Triticum* species, leaf blade (vegetative stage) and anther size (reproductive stage) are two key characters (Rahiminejad and Kharazian, 2005; Salimi *et al*., 2005). The belonging of SSCP profiles of our flawed diploid *Triticum* specimens to one of the two SSCP profiles was confirmed by their complete samples which were provided by growing their seeds. This study proved that SSCP to be a useful tool to separate different taxonomic entities at species level.

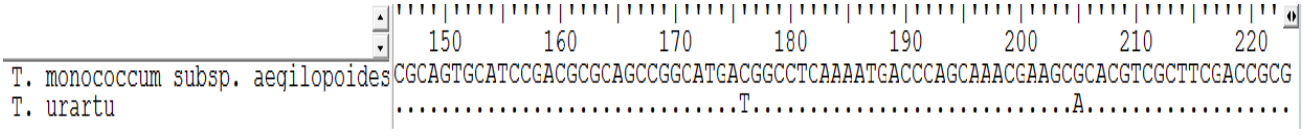

Figure 2. Comparison of ITS2 sequences among wild diploid Triticum material used in this study.

As the final conclusion, this first report of using SSCP among the Iranian *Triticum* materials suggested this method as an effective and distinctive applicable tool for the taxonomy of other plant taxa and represents another step forward toward routine use of DNA sequence data as a tool for identification at the specific level.

Acknowledgement

The authors wish to thank the office of graduate studies of the University of Isfahan for their support.

References

1- Blatter R. H. E., Jacomet S. and Schlumbaum A. (2002) Spelt-specific alleles in HMW glutenin genes from modern and historical European spelt (*Triticum spelta* L.) Theoretical and Applied Genetics 104, 2–3: 329–337.
2- Bodenes C., Laigret F. and Kremer A. (1996) Inheritance and molecular variations of PCR-SSCP fragment in Pedunculate oak (*Quercus robur* L.). Theoretical and Applied Genetics 93, 3: 348–354.
3- Borchert T. and Hohe A. (2009) Identification of molecular markers for the flower type in the ornamental crop *Calluna vulgaris*. Euphytica 170, 1–2: 203–213.
4- Castelblanco W. and Fregene M. (2006) SSCP-SNP-based conserved ortholog set (COS) markers for comparative genomics in cassava (*Manihot esculenta* crantz). Plant Molecular Biology Reporter 24, 2: 229–236.
5- Chen S., Yao H., Han J., Liu C., Song J., Shi L., Zhu, Y., Ma X., Gao T., Pang X., Luo K., Li Y., Li X., Jia X., Lin Y. and Leon C. (2010) Validation of the ITS2 region as a novel DNA Barcode for identifying medicinal plant species. Public Library of Science ONE 5: e8613.
6- Cotton R. G. H. (1997) Slowly but surely towards better scanning for mutations. Trends in Genetics 13, 2: 43–46.
7- Crautlein M., Korpelainen H., Pietilainen M. and Rikkinen J. (2011) DNA barcoding: a tool for improved taxon identification and detection of species diversity. Biodiversity and Conservation 20, 2: 373–389.
8- Dumolin-Lapegue S., Bodenes C. and Petit R. J. (1996) Detection of rare polymorphisms in mitochondrial and of oaks with PCR-RFLP combined to SSCP analysis. Forest Genetics 3, 4: 227–230.
9- Ebihara A., Ishikawa H., Matsumoto S., Lin S. J., Iwatsuki K., Takamiya N., Watano Y. and Ito M. (2005) Nuclear DNA, chloroplast DNA, and ploidy analysis clarified biological complexity of the *Vandesbochia radicans* complex (Hymenophyllaceae) in Japan and adjacent areas. American Journal of Botany 92, 9: 1535−1547.
10- Fukuoka S., Inoue T., Miyao A., Monna L., Zhong H. S., Sasaki T. and Minobe, Y. (1994) Mapping of sequence-tagged sites in rice by single strand conformation polymorphism. DNA Research 1, 6: 271–277.
11- Gasser R. B. and Chilton N. B. (2001) Applications of single-strand conformation polymorphism (SSCP) to taxonomy, diagnosis, population genetics and molecular evolution of parasitic nematodes. Veterinary Parasitology 101, 3–4: 201−213.
12- Gasser R. B., Hu M., Chilton N. B., Campbell B. E., Jex A. J., Otranto D., Cafarchia C., Beveridge I. and Zhu X. (2006) Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. Nature Protocols 1, 6: 3121−3128.
13- Gawel N. J. and Jarret R. L. (1991) A modified CTAB extraction procedure for *Musa* and *Ipomoea*. Plant Molecular Biology Reporter 9, 3: 262–266.
14- Hall T. A. (1999) BioEdit: a user-friendly biological sequence alignment editor analysis program for Windows 95/98/ NT. Nucleic Acids Symposium Series 41: 95–98.
15- Hayashi K. (1991) PCR-SSCP: a simple and sensitive method for detection of mutations in the genomic DNA. PCR methods and applications 1, 1: 34–38.
16- Karatas D. D., Kunter B., Coppola G. and Velasco R. (2010) Analysis of polymorphism based on SSCP markers in gamma-irradiated (Co^{60}) grape (*Vitis vinifera*) varieties. Genetics and Molecular Research 9, 4: 2357−2363.
17- Kita Y., and Ito M. (2000) Nuclear ribosomal ITS sequences and phylogeny in East Asian *Aconitum* subgenus *Aconitum* (Ranunculaceae), with special reference to extensive polymorphism in individual

plants. Plant Systematics and Evolution 225, 1–4: 1–13.
18- Kuhn D. N., Motamayor J. C., Meerow A. W. and Schnell R. J. (2008) SSCP markers provide a useful alternative to microsatellites in genotyping and estimating genetic diversity in populations and germplasm collections of plant specialty crops. Electrophoresis 29, 19: 4096–4108.
19- Lu Y., Curtiss J., Percy R. G., Hughs S. E., Yu S. and Zhang J. (2009) DNA polymorphisms of genes involved in fiber development in a selected set of cultivated tetraploid cotton. Crop Science 49, 5: 1695–1704.
20- Martins-Lopes P., Zhang H. and Koebner R. (2001) Detection of single nucleotide mutations in wheat using single strand conformation polymorphism Gels. Plant Molecular Biology Reporter 19, 2: 159–162.
21- Meusnier I., Valero M., Destombe C., Gode C., Desmarais E., Bonhomme F., Stam W. T. and Olsen J. (2002) PCR-SSCP analyses of nuclear and chloroplast DNA provide evidence for recombination, multiple introductions and nascent speciation in the *Caulerpa taxifolia* complex. Molecular Ecology 11: 2317–2325.
22- Olivares-Fuster O., Hernandez-Garrido M., Guerri J. and Navarro L. (2007) Plant somatic hybrid cytoplasmic DNA characterization by single-strand conformation polymorphism. Tree Physiology 27, 6: 785–792.
23- Orita M., Suzuki Y., Sekiya T. and Hayashi K. (1989) Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5, 4: 874–879.
24- Plomion C., Hurme P., Frigerio J. M., Ridolfi M., Pot D. and Pionneau C. (1999) Developing SSCP markers in two *Pinus* species. Molecular Breeding 5, 1: 21–31.
25- Rahiminejad M. R. and Kharazian N. (2005) Illustration of and a key to the genus *Triticum* L. in Iran. Wheat information Service 99: 29–34.
26- Rodriguez F., Cai D., Teng Y. and Spooner D. (2011) Asymmetric single-strand conformation polymorphism: an accurate and cost-effective method to amplify and sequence allelic variants. American Journal of Botany 98, 7: 1061–1067.
27- Rousseau-Gueutin M., Gaston A., Ainouche A., Ainouche M. L., Olbricht K., Staudt G., Richard L. and Denoyes-Rothan B. (2009) Tracking the evolutionary history of polyploidy in *Fragaria* L. (strawberry): New insights from phylogenetic analyses of low-copy nuclear genes. Molecular Phylogenetics Evolution 51, 3: 515–530.
28- Salimi A., Ebrahimzadeh H. and Taeb M. (2005) Description of Iranian diploid wheat resources. Genetic Resources and Crop Evolution 52, 4: 351–361.
29- Salmaso M., Faes G., Segala C., Stefanini M., Salakhutdinov I., Zyprian E., Toepfer R., Grando M. S. and Velasco R. (2004) Genome diversity and gene haplotypes in the grapevine (*Vitis vinifera* L.), as revealed by single nucleotide polymorphisms. Molecular Breeding 14, 4: 385–395.
30- Sanguinetti C. J., Dias Neto E. and Simpson A. J. G. (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. Biotechniques 17, 5: 915–919.
31- Sato Y. and Nishio T. (2003) Mutation detection in rice waxy mutants by PCR-RF-SSCP. Theoretical and Applied Genetics 107, 3: 560–567.
32- Sunnucks P., Wilson A. C. C., Beheregaray L. B., Zenger K., French J. and Taylor A. C. (2000) SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. Molecular Ecology 9, 11: 1699–1710.
33- Liberado P. and Rozas J. (2009) DnaSP version 5.10: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25: 1451–1452.
34- Swapna M., Sivaraju K., Sharma R. K., Singh N. K. and Mohapatra T. (2011) Single strand conformational polymorphism of EST-SSRs: A potential tool for diversity analysis and varietal identification in sugarcane. Plant Molecular Biology Reporter 29, 3: 505–513.
35- To K. Y., Liu C. I., Liu S. T. and Chang Y. S. (1993) Detection of point mutations in the chloroplast genome by single-strand conformation polymorphism analysis. The Plant Journal 3, 1: 183–186.
36- Wang G. Z., Miyashita N. T. and

Tsunewaki K. (1997) Plasmon analyses of *Triticum* (wheat) and *Aegilops*: PCR-single-stranded conformational polymorphism (PCR-SSCP) analyses of organellar DNAs. Proceedings of the National Academy of Science of USA 94, 26: 14570–14577.

37- Watano Y., Imazu M. and Shimizu T. (1995) Chloroplast DNA typing by PCR-SSCP in the *Pinus pumila*, *P. parviflora* var. *pentaphylla* complex (Pinaceae). Journal of Plant Research 108, 4: 493–499.

38- Watano Y., Kanai A. and Tani N. (2004) Genetic structure of hybrid zones between *Pinus pumila* and *P. parviflora* var. *pentaphylla* (Pinaceae) revealed by molecular hybrid index analysis. American Journal of Botany 91, 1: 65–72.

39- White T. J., Bruns T. D., Lee S. B. and Taylor J. W. (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR-protocols and applications-a laboratory manual. (Eds. Innis, N., Gelfand, D., Sninsky, J. and White, J.) Academic Press, New York, 315–322 pp.

40- Yao H., Song J., Liu C., Luo K., Han J., Li Y., Pang P., Xu H., Zhu Y., Xiao P. and Chen S. (2010) Use of ITS2 Region as the Universal DNA Barcode for Plants and Animals. Public Library of Science ONE 5: e13102.

Mini-Review

ATP-binding cassette (ABC) transporters as emerging targets in modulation of neural stem cells behavior in neurodegenerative diseases and cell therapy benefits

Sandeep Kumar Vishwakarma[1,2], Syed Ameer Basha Paspala[1,2], Santosh K Tiwari[1], Aleem A Khan*[1,2]

1- Central Laboratory for Stem Cells & translational Medicine, Centre for Liver Research and Diagnostics, Deccan College of Medical Sciences, Kanchanbagh, Hyderabad, 500 058, Andhra Pradesh, India
2-PAN Research Foundation, Narayanguda, Hyderabad, Andhra Pradesh, India

Received 01 Jan 2014 Accepted 16 Feb 2014

Abstract

Increasing incidence of central nervous system (CNS) disorders has become a major challenge for both basic and clinical scientific society to develop novel therapeutic models for treatment. The knowledge of stem cells has added a new dimension in the research towards finding more appropriate targets responsible for the disease fate determination. As stem cell research is progressing day by day in routine research laboratories there is great hope to find suitable therapeutic targets for complete cure of the CNS disorders. Discovery of ABC transporters in animal tissues has emerged as new spot for several disease prognosis and therapeutic target. ABC transporters are membrane proteins expressed in various organs like liver, kidney, blood-brain barrier, blood-testis barrier etc. It is involved in various important cellular processes such as absorption, distribution and excretion of drugs, xenobiotics and endogenous compounds showing their role in tissue defense and organ regeneration. The current review explains about the role of ABC transporters in CNS pathogenesis and defense adding stem cells therapeutic strategies.

Keywords: ABC transporters; Neurodegenerative diseases; Stem cell therapy

Introduction

ABC transporters are ATP-dependent membrane proteins which are expressed in a variety of organs, such as the kidney, liver, placenta, intestine, blood-testes barrier and blood-brain barrier. In these organs ABC transporters and its members play vital role in many cellular processes such as absorption, distribution, and excretion of various types of drugs, toxins, xenobiotics, and endogenous compounds (Fig. 1). Based on the sequence homology, order of domain and similarity in gene structures 49 members of human ABC transporters have been identified and are divided into seven subfamilies from A to G. Although few ABC transporters have been studied comprehensively in bacteria, yeast and other organisms, the functional annotation of many others are still need to identify. Mostly identified ABC transporters have two types of primary structures 1) full transporters and 2) half transporters. Each have different way of interactions with the molecules and are most important component in defense mechanisms (Fig. 2). With the disease perspectives 16 ABC genes have been identified linked to inherited diseases, such as Tangier disease (ABCA1), Dubin Johnson syndrome (ABCC2), *pseudoxanthoma elasticum* (ABCC6), and cystic fibrosis (ABCC7) (Dean, 2005). Development of Knockout animal models for ABC transporters has provided us with some insights into the function and characteristics of ABC transporters (Schinkel et al., 1997; Xia et al., 2007; Glaeser and Fromm, 2008). The knowledge of neural stem cells (NSCs) has added a new dimension for the study of CNS regeneration based on cellular and molecular depiction. However, a variety of molecular targets are being identified involved in CNS damage and regeneration, none of them have proved their potential as appropriate prognostic and therapeutic targets. Hence, there is need to identify more appropriate molecular switches that is responsible for specific disease condition and can be targeted to improve the cell therapy benefits. In this scenario targeting the molecular pathways involved in NSCs proliferation and differentiation would probably help to identify the defined target for CNS regeneration.

*Corresponding author E-mail:
aleem_a_khan@rediffmail.com

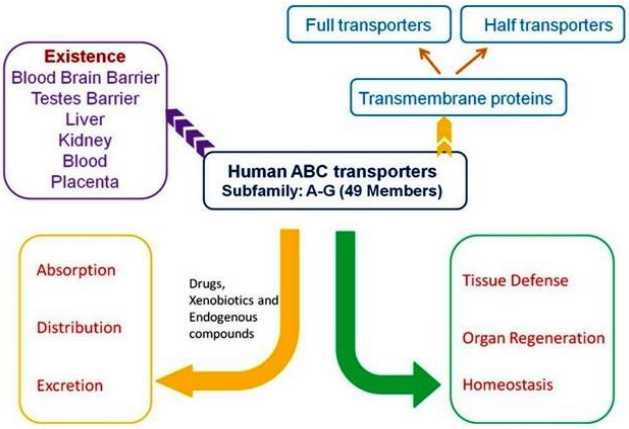

Fig 1 Schematic representation of human ABC transporters existence and their role in tissue defense and organ regeneration by excretion, absorption and distribution of toxic substances within/out of the cells.

NSCs are specialized cell populations within the central nervous system (CNS) tissue and characterized as self-renewing and multipotent in nature having capacity to generate neurons and glia upon differentiation. They can be isolated, genetically manipulated and differentiated in vitro retaining its ability to reintroduce in cell replacement therapies for the treatment of various neurodegenerative diseases. During differentiation characterization of genes with tightly controlled expression patterns signifies extensive approach to understand the regulatory behavior of stem cells.

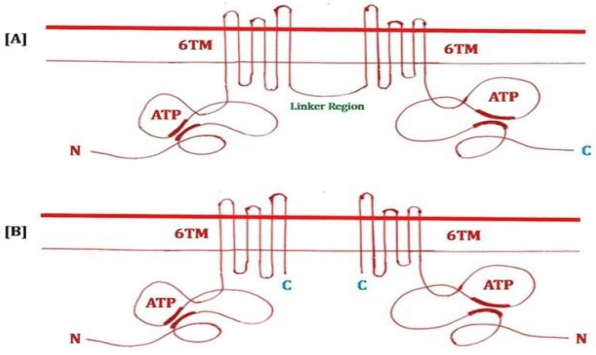

Fig 2: Two primary types of membrane associated ABC transporter proteins [A] Full transporter shows mirror image halves separated by a flexible linker region essential for catalytic activity. Each half of the molecule is a 6-transmembrane spanning domain (6TM) that blocks the transporter proteins to the plasma membrane [B] Half transporters are consist of only one halve and require membrane association with other half transporter to form the pure complex. There is no linker region present in the half transporter proteins.

The regulation of NSCs behavior in vitro and in vivo by using ABC transporters has emerged an essential innovative meadow of investigation as major focus on their integrated analysis in NSCs proliferation, differentiation and regulation, along with comparison to that in hematopoietic and other stem cells (To Kenneth et al., 2011; Ishimoto et al., 2014).

ABC transporters and stem cells

Stem cells have become a major target of interest for identifying molecules with tightly controlled expression during proliferation and differentiation in order to get more suitable molecular target to understand the disease pathogenesis and tissue defense mechanisms (Fig. 3). In this perspective, ABC transporters represent a strong applicant to be studied in more comprehensive way to understand their role in stem cells proliferation and differentiation. Discovery of P-gp on CD34 positive hematopoietic cells by Chaudhari and Roninson (1991) emerged a new insight for separate category of molecule to study in disease perspectives. Ten years later, expression and functional activity of BCRP in these cells was identified by Zhou et al., (2001). They are normally characterized by their low retention of rhodamine 123, transported by P-gp or BCRP (Litman et al., 2000; Honjo et al., 2001), and Hoechst dye 33342, transported by BCRP in the cells and termed as side population (SP) cells (Golebiewska et al., 2011).

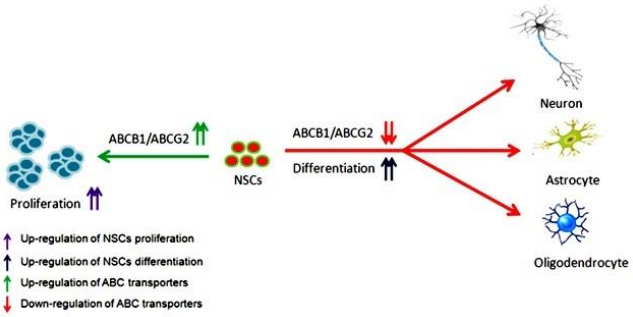

Fig 3: Role of ABC transporters in NSCs proliferation and differentiation. Usually during NSCs proliferation expression of ABC transporters increases and decreases during differentiation

Now, it is well known that the SP population cells are not only resides within the bone marrow but also in other nonhematopoietical organs, such as umbilical cord blood, brain, spleen tissue, kidney, heart, skin, intestine, and lungs (Asakura and Rudnicki, 2002; Yano et al., 2005; Larderet et al., 2006). In these organs, they have ability to differentiate into several cell types following re-introduction in vivo, including neurons, cardiac and skeletal muscles, and epithelial cells like hepatocytes (Ferrari et al., 1998; Alison et al., 2000; Lagasse et al., 2000; Mezey et al., 2000; Krause et al., 2001; Jackson et al., 2001).

ABC transporters in tissue defense and organ regeneration

Various studies have demonstrated the significant role of various ABC transporters in tissue defense through the excretion of toxic compounds and their metabolites (Russel et al., 2002; Szaka´cs et al., 2008). The transporters are expressed in highly controlled manner, emphasizing their consequences in organ protection (Leslie et al., 2005). Two most studied ABC transporters, the multidrug resistance gene 1 product (MDR1/ABCB1), P-glycoprotein (P-gp), and ABCG2 or breast cancer resistance protein 1 (BCRP), have been found to be implicated in tissue regeneration (Padmanabhan et al., 2012). Both the efflux pumps are highly expressed on side population (SP) cells in a variety of tissues. Loss of expression for transporter genes leads to cell differentiation, indicating that they might determine stem cell-induced tissue remodeling through their differential expression. SP cells have been demonstrated for their implication in organ regeneration (Jackson et al., 1999; Lagasse et al., 2000; Jackson et al., 2001).

The localization of ABC transporters within the organ with a barrier function, and the broad substrate specificities imply their role in tissue defense. In brain drug distribution is hampered by few ABC transporters like P-gp, multidrug resistance proteins 1 and 2 (MRP1/2; ABCC1/2), MRP4 (ABCC4), and BCRP in the blood-brain barrier (Kusuhara and Sugiyama, 2001; Begley, 2004; Perriere et al., 2007). The defense mechanism formed by ABC transporters under physiological conditions is intended against accumulation of potentially harmful compounds. Interestingly, during organ damage or disease conditions, changes in the gene expression levels of ABC transporters have been observed, probably to balance for the increased consignment of detrimental products of oxidative stress formed during an insult or to compensate for the loss of efflux pumps in damaged tissues (Mangum et al., 2011; Abdullah et al., 2013).

ABC transporters in human neurodegenerative diseases

In 1976, when Drs. Juliano and Ling reported the overexpression of membrane proteins in colchicine-resistant Chinese hamster ovary cells they found the acquired resistance to many structurally unrelated antineoplastic drugs. The over expressed nmembrane protein was later determined to be a member of ABC transporter superfamily. The ABC domains of these proteins hydrolyze ATP, providing energy for transport of various substrates across the membrane against a high concentration gradient.

Now most highlighted issues of current pharmaceutical biotechnology and cell therapy contains several reviews that summarize the role of the ABC transporters in cellular and molecular therapy as well as in protecting healthy tissue from the xenobiotics and other toxic compounds by modulating various transcription factors and genes. Robey et al. (2001) demonstrated the clinical interventions with ABCG2. Two in-depth articles on the latest important developments in utilizing therapeutic strategies to overcome ABC transporter-mediated drug resistance are reviewed by C-P. Wu et al. 2008 (Natural product modulators of ABC drug transporters) and Stolarczyk et al. 2011 (Targeting phosphorylation to regulate ABC transporter function).

Another important pharmacological / physiological function of ABC transporters is to provide protection by effluxing xenobiotics (including drugs used to treat diseases) from tissues including kidney, testes, brain, and developing human fetus. Hartz and. Bauer (2010) summarized the recent findings on ABC transporter regulation in the CNS especially at the blood-brain barrier and discuss the role of ABC transporters in CNS diseases, including seizures, epilepsy, brain cancer, and alzheimer's disease. Another study by Ni and Mao (2011) provided a thorough discussion of the protective roles of ABC transporters in the placenta that attenuates toxicity in the developing fetus.

ABC transporters and stem cell therapeutic approaches for human neurodegenerative disorders–how to make it work

ABC transporters play a crucial role in several physiological barriers such as the blood brain barrier, blood-cerebrospinal fluid (B-CSF) barrier, and blood-testis barrier modulating the absorption and excretion of xenobiotics across these barriers. Within the CNS, these transporters are localized at the luminal membrane of endothelial cells of blood capillaries where they actively modulate the permeation of xenobiotics. The over-expression of several transporters has been observed in various types of tumors causing multidrug resistance (MDR) to treatment with chemotherapeutic agents (Gangavarapu et al., 2013). Moreover, resistance to CNS drugs, such as antidepressant, antiepileptic and anti-HIV medicine, may also be related to over expression of ABC transporters. Recently, it has been reported that alterations in ABC expression and function are related to the etiology and pathogenesis of neurologic disorders, such as alzheimer’s disease and parkinson’s disease.

However, a recent study reported that ABC expression and function are strongly decreased during the neuro-inflammation process in multiple sclerosis and neuroblastoma (Ingram et al., 2013).

Recent progresses in stem cell biology and therapeutic interventions there is a hope to get the cellular therapies for several human neurodegenerative diseases (Solomon et al., 2012). However, before initiation of their use in clinical trials, we should be able to control stem cell proliferation and differentiation into specific cell phenotype, proper induction of their integration into existing neural and synaptic circuits, and optimized functional recovery in animal models closely resembling to the human diseases.

Recent and future challenges for cellular therapeutic benefits

As with any new medical intrusion, development of stem-cell therapies must awe the scientific and ethical guidelines of human testing. There is need to answer the following questions before claiming the triumph in using stem cells to treat neurodegenerative diseases:

- Types of stem cells used in different diseases.
- Suitable route for stem cells delivery to make functional acquaintances with the host cells.
- Safety and efficacy of stem cells delivery into people with debilitating diseases.
- Applicability of immunosuppressive drugs to prevent the immune rejection of implanted cells.
- Toxic effects and tolerable ability of the patients against these drugs.
- Potential long-term complications of stem-cell therapies.
- Migration of transplanted cells to its original niche of damaged tissue.
- Tumor development.
- Progression of the disease condition, functional improvements, or speed recovery for patients with diseases.
- Modulation of stem cells transcription factors and other proteins playing significant roles in stem cells proliferation and differentiation.

It is an exciting time, but we must move forward with meticulous regard for the scientific process and cautious respect for what we do not know and can't anticipate. The answers to the myriad questions are needed to be addressed. With the guidance and assistance of regulatory agencies, scientists and clinicians from different areas must work together to make this hope a reality.

Next steps toward cell therapy and repair

Further research is needed in order to prove its ability to coax endogenous stem cells in the adult nervous system to respond in better way to the injury. It will also provide the acceptable cells for transplantation after modifying gene expression patterns further to ameliorate different types of diseases. The types of nervous system diseases that represent the best targets for stem cell–based therapies are those that would be improved by the transplant or induced replacement of a limited number of cell types. Sensory disorders, parkinson's and glial diseases fall into this category and could potentially be cured by a cell-replacement therapy by modifying the expression of ABC transporters and HSPs. Motor system disorders and spinal cord injuries are more complex, but given their severity and lack of current treatment options, it can be argued that any improvement in function would be of great benefit. Drug transporters and HSPs expression analysis by various studies have demonstrated their role in such diseases and may provide a better target to settle the treatment strategies (Schumacher et al., 2012; Zheng et al., 2013; Alberto et al., 2014).

The hope of using stem cells to intervene in neurodegenerative disease is promising, but due to various complexities of the central nervous system, advancements will likely continue in deliberate steps. Further to move the research forward, it is critical to measure the efficacy of any experiment involving human and animal subjects. Despite the molecular differences between neurodegenerative diseases, their eventual stem cell therapies will likely share many features such as information gained in one field can drive forward progress in the others.

Acknowledgement

We are highly thankful for Indian Council of Medical Research, New Delhi, India for providing support to start the study on human neural stem cells

Conflict of interest

The authors declare no conflict of interest

References

1. Abdullah LN and Chow EK 2013 Mechanisms of chemoresistance in cancer stem cells. Clinic. & Translat. Med. 2 (3).
2. Alberto L, Fabiana L, Sandra C, Vicente C and Analía T 2014 Stem-cell marker CD34, multidrug resistance proteins P-gp and

BCRP in SEGA. Recept. & Clinic. Invest. 1.
3. Alison MR, Poulsom R, Jeffery R, Dhillon AP and Quaglia A, et al 2000 Hepatocytes from non-hepatic adult stem cells. Nat. 406 257.
4. Asakura A and Rudnicki MA 2002 Side population cells from diverse adult tissues are capable of in vitro hematopoietic differentiation. Exp. Hematol. 30 1339–1345.
5. Begley DJ 2004 ABC transporters and the blood-brain barrier. Curr. Pharm. Des. 10 1295–1312.
6. Chaudhary PM and Roninson IB 1991 Expression and activity of P-glycoprotein, a multidrug efflux pump, in human hematopoietic stem cells. Cell. 66 85–94.
7. Chung-Pu Wu, Anna Maria Calcagno, and Suresh VA 2008 Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: Evaluation of current strategies. Curr. Mol. Pharmacol. 2008 1(2) 93–105.
8. Dean M 2005 The genetics of ATP-binding cassette transporters. Meth. Enzymol. 400 409–429
9. Elzbieta IS, Cassandra JR, and Christian MP 2011 Regulation of ABC Transporter Function Via Phosphorylation by Protein Kinases. Curr. Pharm. Biotechnol. 1 12(4) 621–635.
10. Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, Cossu G, and Mavilio F 1998 Muscle regeneration by bone marrow-derived myogenic progenitors. Sci. 279 1528–1530.
11. Gangavarapu KJ, Azabdaftari G, Morrison CD, Miller A and Foster BA, et al 2013 Aldehyde dehydrogenase and ATP binding cassette transporter G2 (ABCG2) functional assays isolate different populations of prostate stem cells where ABCG2 function selects for cells with increased stem cell activity. Stem Cell Res. Ther. 4 (5) 132.
12. Glaeser H and Fromm MF 2008 Animal models and intestinal drug transport. Exp. Opin. Drug Metab. Toxicol. 4 347–361.
13. Golebiewska A, Brons NHC, Bjerkvig R and Niclou SP 2011 Critical Appraisal of the Side Population Assay in Stem Cell and Cancer Stem Cell Research. Cell Stem Cell. 8 136-147.
14. Hartz AMS and Bauer B 2010 Regulation of ABC transporters at the blood-brain barrier: New targets for CNS therapy. Mol. Interv. 10 5.
15. Honjo Y, Hrycyna CA, Yan QW, Medina-Pe´rez WY and Robey RW, et al 2001 Acquired mutations in the MXR/BCRP/ABCP gene alter substrate specificity in MXR/BCRP/ABCP-overexpressing cells. Canc. Res. 61 6635–6639.
16. Ingram WJ, Crowther LM, Little EB, Freeman R and Hallahan AR et al 2013 ABC transporter activity linked to radiation resistance and molecular subtype in pediatric medulloblastoma. Exp. Hematol. Oncol. 2 (26).
17. Ishimoto T, Nakamichi N, Hosotani H, Masuo Y and Sugiura T, et al 2014 Organic Cation Transporter-Mediated Ergothioneine Uptake in Mouse Neural Progenitor Cells Suppresses Proliferation and Promotes Differentiation into Neurons. PLoS ONE. 9 (2).
18. Jackson KA, Majka SM, Wang H, Pocius J and Hartley CJ, et al 2001 Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. J. Clin. Invest. 107 1395–1402.
19. Jackson KA, Mi T, and Goodell MA 1999 Hematopoietic potential of stem cells isolated from murine skeletal muscle. Proc. Natl. Acad. Sci. U. S. A. 96 14482–14486.
20. Krause DS, Theise ND, Collector MI, Henegariu O and Hwang S, et al 2001 Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell. 105 369–377.
21. Kusuhara H and Sugiyama Y 2001 Efflux transport systems for drugs at the blood-brain barrier and blood-cerebrospinal fluid barrier (part 1). Drug. Discov. Today. 6 150–156.
22. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M and Dohse M, et al 2000 Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat. Med. 6 1229–1234.
23. Larderet G, Fortunel NO, Vaigot P, Cegalerba M and Malte`re P, et al 2006 Human side population keratinocytes exhibit long-term proliferative potential and a specific gene expression profile and can form a pluristratified epidermis. Stem Cells. 24 965–974.

24. Leslie EM, Deeley RG, and Cole SP 2005 Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol. Appl. Pharmacol. 204 216–237.
25. Litman T, Brangi M, Hudson E, Fetsch P and Abati A, et al 2000 The multidrug-resistant phenotype associated with overexpression of the new ABC half-transporter, MXR (ABCG2). J. Cell. Sci. 113 2011–2021.
26. Mangum R, Nakano I 2011 Glioma Stem Cells and their Therapy Resistance. J Carcinogene Mutagene S1:002.
27. Mezey E, Chandross KJ, Harta G, Maki RA, and McKercher SR 2000 Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. Sci. 290 1779–1782.
28. Ni Z and Mao Q 2011 ATP-binding cassette efflux transporters in human placenta. Curr. Pharm. Biotechnol. 12(4) 674-685.
29. Padmanabhan R, Chen KG, Gillet JP, Handley M and Mallon BS, et al 2012 Regulation and Expression of the ATP-Binding Cassette Transporter ABCG2 in Human Embryonic Stem Cells. Stem Cells 30 (10) 2175–2187.
30. Perrie`re N, Yousif S, Cazaubon S, Chaverot N and Bourasset F, et al 2007 A functional in vitro model of rat blood-brain barrier for molecular analysis of efflux transporters. Brain. Res. 1150 1–13.
31. Russel FG, Masereeuw R, and Van Aubel RA 2002 Molecular aspects of renal anionic drug transport. Ann. Rev. Physiol. 64 563–594.
32. Schinkel A. H. 1997 The physiological function of drug-transporting P-glycoproteins. Semin. Canc. Biol. 8 161–170
33. Schumacher T, Krohn M, Hofrichter J, Lange C and Stenzel J, et al. 2012 ABC Transporters B1, C1 and G2 Differentially Regulate Neuroregeneration in Mice. PLoS ONE. 7(4) e35613.
34. Solomon OU and Jian T 2012 Bench to Bedside of Neural Stem Cell in Traumatic Brain Injury. Stem Cells Internat. 8.
35. Szaka´cs G, Va´radi A, Ozvegy-Laczka C, and Sarkadi B 2008 The role of ABC transporters in drug absorption, distribution, metabolism, excretion and toxicity (ADME-Tox). Drug. Discov. Today. 13 379–393.
36. To Kenneth KW and Fu LW 2011 Multidrug Resistance Transporters – Roles in maintaining Cancer Stem-Like Cells, Stem Cells in Clinic and Research, Dr. Ali Gholamrezanezhad (Ed.), InTech.
37. Xia CQ, Milton MN, and Gan LS 2007 Evaluation of drug-transporter interactions using in vitro and in vivo models. Curr. Drug Metab. 8 341–363.
38. Yano S, Ito Y, Fujimoto M, Hamazaki TS, Tamaki K, and Okochi H 2005 Characterization and localization of side population cells in mouse skin. Stem Cells. 23 834–841.
39. Zheng X, Naiditch J, Czurylo M, Jie C and Lautz T, et al 2013 Differential effect of long-term drug selection with doxorubicin and vorinostat on neuroblastoma cells with cancer stem cell characteristics. Cell Death and Disease. 4 e740.
40. Zhou S, Schuetz JD, Bunting KD, Colapietro AM and Sampath J, et al 2001 The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat. Med. 7 1028–1034.

Scientific Reviewers

MANUSCRIPT PREPARATION

Manuscripts should be prepared in accordance with the uniform requirements for Manuscript's Submission to **"Journal of Cell and Molecular Research".**

Language: Papers should be in English (either British or American spelling). The past tense should be used throughout the results description, and the present tense in referring to previously established and generally accepted results. Authors who are unsure of correct English usage should have their manuscript checked by somebody who is proficient in the language; manuscripts that are deficient in this respect may be returned to the author for revision before scientific review.

Typing: Manuscripts must be typewritten in a font size of at least 12 points, double-spaced (including References, Tables and Figure legends) with wide margins (2.5 cm from all sides) on one side of the paper. The beginning of each new paragraph must be clearly indicated by indentation. All pages should be numbered consecutively at the bottom starting with the title page.

Length: The length of research articles should be restricted to ten printed pages. Short communication should not exceed five pages of manuscript, including references, figures and tables. Letters should be 400-500 words having 7-10 references, one figure or table if necessary. Commentaries and news should also be 800-1000 words having 7-10 references and one figure or table if necessary.

Types of Manuscript: JCMR is accepting original research paper, short communication reports, invited reviews, letters to editor, biographies of scientific reviewers, commentaries and news.

GENERAL ARRANGEMENT OF PAPERS

Title: In the first page, papers should be headed by a concise and informative title. The title should be followed by the authors' full first names, middle initials and last names and by names and addresses of laboratories where the work was carried out. Identify the affiliations of all authors and their institutions, departments or organization by use of Arabic numbers (1, 2, 3, etc.).

Footnotes: The name and full postal address, telephone, fax and E-mail number of corresponding author should be provided in a footnote.

Abbreviations: The Journal publishes a standard abbreviation list at the front of every issue. These standard abbreviations do not need to be spelled out within paper. However, non-standard and undefined abbreviations used five or more times should be listed in the footnote. Abbreviations should be defined where first mentioned in the text. Do not use abbreviations in the title or in the Abstract. However, they can be used in Figures and Tables with explanation in the Figure legend or in a footnote to the Table.

Abstract: In second page, abstract should follow the title (no authors' name) in structured format of not more than 250 words and must be able to stand independently and should state the Background, Methods, Results and Conclusion. Write the abstract in third person. References should not be cited and abbreviations should be avoided.

Keywords: A list of three to five keywords for indexing should be included at bottom of the abstract. Introduction should contain a description of the problem under investigation and a brief survey of the existing literature on the subject.

Materials and Methods: Sufficient details must be provided to allow the work to be repeated. Correct chemical names should be given and strains of organisms should be specified. Suppliers of materials need only be mentioned if this may affect the results. Use System International (SI) units and symbols.

Results: This section should describe concisely the rationale of the investigation and its outcomes. Data should not be repeated in both a Table and a Figure. Tables and Figures should be selected to illustrate specific points. Do not tabulate or illustrate points that can be adequately and concisely described in the text.

Discussion: This should not simply recapitulate the Results. It should relate results to previous work and interpret them. Combined Results and Discussion sections are encouraged when appropriate.

Acknowledgments: This optional part should include a statement thanking those who assisted substantially with work relevant to the study. Grant support should be included in this section.

References: References should be numbered and written in alphabetical order. Only published, "in press" papers, and books may be cited in the reference list (see the examples below). References to work "in press" must be accompanied by a copy of acceptance letter from the journal. References should not be given to personal communications, unpublished data, manuscripts in preparation, letters, company publications, patents pending, and URLs for websites. Abstracts of papers presented at meetings are not permissible. These references should appear as parenthetical expressions in the text, e.g. (unpublished data). Few example of referencing patterns are given as follows:

Bongso A., Lee E. H. and Brenner S. (2005) Stem cells from bench to bed side. World Scientific Publishing Co. Singapore, 38-55 pp.

Irfan-Maqsood, M. (2013) Stem Cells of Epidermis: A Critical Introduction. Journal of Cell and Molecular Research 5(1): 1-2.

Note: All the reference should be in EndNote format (JCMR EndNote Style is available on JCMR's web site, Author's Guideline)

Tables and Figures: Tables and Figures should be numbered (1, 2, 3, etc.) as they appear in the text. Figures should preferably be the size intended for publication. Tables and Figures should be carefully marked. Legends should be typed single-spaced separately from the figures. Photographs must be originals of high quality. Photocopies are not acceptable. Those wishing to submit color photographs should contact the Editor regarding charges.

Black Page Charges: There is no black page charges for publication in the Journal of Cell and Molecular Research.

Color Page Charges: All color pages being printed in color will cost 1,000,000 Iranian Rials/page.

Table of Contents

Journal of Cell and Molecular Research

Volume 6, Number 1, Summer 2014