



# Syndromic and non-syndromic forms of retinitis pigmentosa: a comprehensive Italian clinical and molecular study reveals new mutations

C.O. Pierrottet<sup>1</sup>, M. Zuntini<sup>2</sup>, M. Digiuni<sup>1</sup>, I. Bazzanella<sup>2</sup>, P. Ferri<sup>1</sup>, R. Paderni<sup>1</sup>, L.M. Rossetti<sup>1</sup>, S. Cecchin<sup>2</sup>, N. Orzalesi<sup>1</sup> and M. Bertelli<sup>2</sup>

<sup>1</sup>Department of Ophthalmology, San Paolo Hospital, University of Milan, Milan, Italy

<sup>2</sup>MAGI Human Medical Genetics Institute, Rovereto, Italy

Corresponding author: C.O. Pierrottet  
E-mail: [chiarapierrottet@tiscali.it](mailto:chiarapierrottet@tiscali.it)

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**ABSTRACT.** Mutations in more than 60 different genes have been associated with non-syndromic and syndromic retinitis pigmentosa (RP), a heterogeneous group of inherited retinal dystrophies. To increase the understanding of the molecular epidemiology of the disease in Italy, we analyzed 56 patients with syndromic and non-syndromic forms of RP attending the Retinitis Pigmentosa Center of San Paolo Hospital (Milan, Italy). Patients underwent detailed clinical examination. Genomic DNA isolated from peripheral blood samples was screened for mutations in different genes according to RP form by direct sequencing analysis. The impact of novel missense mutations on protein functions was predicted by *in silico* analysis and protein sequence alignment. Cosegregation analysis was performed between available family members. Forty-one of the 56 probands analyzed had non-syndromic and 15 had syndromic RP forms. Putative disease-causing mutations were identified in 19 of 56 unrelated RP

probands. Mutation screening identified a total of 22 different heterozygous variants. Notably, 12 of these putative pathogenic mutations have not been previously reported. New variants were found to be located on the *USH2A*, *RPGR*, *EYS*, and *RHO* genes. All 3 new variants detected in X-linked RP probands were confirmed in other affected family members. We found a positivity rate of 24.4% and 60% for probands with non-syndromic and syndromic RP, respectively. This is the first report of *RPGR* X-linked RP proband-ORF15 mutations in Italian patients with X-linked (XL)-RP. In addition, this is the first report of data regarding the association between *EYS* mutations and non-syndromic RP forms in the Italian population.

**Key words:** Bardet-Biedl syndrome; Molecular screening; Retinitis pigmentosa; Usher syndrome

## INTRODUCTION

Retinitis pigmentosa (RP; OMIM 268000) is a heterogeneous group of inherited retinal dystrophies characterized by photoreceptor and retinal pigment epithelium abnormalities that lead to progressive retinal degeneration, atrophy, and vision loss (Chang et al., 2011). RP is one of the leading causes of blindness in adults, with a prevalence of approximately 1 in 3500-5000 individuals worldwide (Rivolta et al., 2002). Typical symptoms include night blindness, progressive peripheral visual field loss (often leading to tunnel vision), and, in later stages, visual acuity impairment. Clinical hallmarks include an abnormal fundus with bone-spicule pigment deposits and attenuated retinal vessels, abnormal, diminished, or absent electroretinographic findings, and reduced visual fields. Symptoms typically start during the early teenage years, and severe visual impairment occurs by 40-50 years of age. There are also early-onset, late-onset, and even nonpenetrant forms of RP (Daiger et al., 2007).

RP can be subdivided into non-syndromic RP, without systemic abnormalities, and syndromic RP, in which retinal degeneration is associated with other symptoms due to systemic diseases. The most frequent syndromic form of RP is Usher syndrome (OMIM #276900-902), which manifests as early-onset or congenital neurosensory hearing impairment followed by development of RP (Kimberling et al., 2000; Keats and Savas, 2004). The second most common syndromic form is Bardet-Biedl syndrome (OMIM #209900), which includes RP, polydactyly, obesity, renal abnormalities, hypogenitalism, and mental retardation (Katsanis, 2004). Many other complex, pleiotropic conditions include RP as a component (Daiger et al., 2007).

Mutations in more than 60 different genes associated with non-syndromic and syndromic RP phenotypes have been described (RetNet, 2013 database: <https://sph.uth.edu/retnet/>).

Non-syndromic RP can be inherited in an autosomal dominant (adRP), recessive (arRP), or X-linked (XL-RP) manner. Rare cases of RP presenting mitochondrial (Mansergh et al., 1999) or digenic inheritance (Kajiwara et al., 1994), uniparental disomy, and incomplete penetrance (Rivolta et al., 2002) have also been described. A number of cases cannot be classified and are therefore referred to as isolated or sporadic RP (sRP; single occurrence in a family) (Pagon and Daiger, 2000).

adRP accounts for 15-25% of all RP cases (Pagon and Daiger, 2000; Daiger et al., 2007), depending on the country and the ethnic group analyzed. Two studies reported a prevalence of approximately 17% for adRP in the Italian population (Fossarello et al., 1993; Ziviello

et al., 2005), which agrees with the results of other studies carried out in Southern Europe (Ayuso et al., 1995). The genes typically associated with adRP include *RHO* (25-30%), *PRPF31* (15-20%), *PRPH2* (5-10%), *RPI* (5-10%), *PRPF3*, and *IMPDHI* (3-5%) (Pagon and Daiger, 2000; Fahim et al., 2013).

arRP is estimated to account for 5-20% of all forms of non-syndromic RP (Pagon and Daiger, 2000; Daiger et al., 2007). The genes most frequently affected in arRP patients include *EYS* and *RPE65* (Pagon and Daiger, 2000).

In addition, 5-15% of RP patients show X-linked inheritance (XL-RP) (Pagon and Daiger, 2000), which is generally the most severe form, characterized by early onset and rapid progression. Two genes, *RP2* and *RPGR*, account for 10-20 and 70-90% of cases, respectively (Vervoort et al., 2000; Fahim et al., 2013).

The high degree of genotypic and phenotypic variability, including within families, as well as the current lack of specific treatments, make customized management necessary. These treatments should consider clinical, genetic, rehabilitative, and psychological aspects (Parmeggiani et al., 2011). In this context, accurate molecular screening enables genotype-phenotype correlation studies, which are useful for predicting disease severity and for effective genetic counseling. Moreover, since gene therapy prospects have improved for a variety of retinal disorders, including RP (Liu et al., 2011; Beltran et al., 2012), genetic screening results can provide important information that can be used to develop innovative treatments.

Here, we present the results of clinical and genetic characterization of 56 Italian probands with syndromic and non-syndromic forms of RP, contributing to the elucidation of RP molecular epidemiology in Italy. We identified a total of 22 different causative mutations in 19 patients and describe 12 new mutations that were further characterized for their putative pathogenic potential.

## MATERIAL AND METHODS

### Subjects and clinical evaluation

Fifty-six consecutive probands with clinically suspected RP and their family members were examined at the University Eye Clinic, Health Department, San Paolo Hospital (Milan, Italy) from May 2011 to October 2012. All RP families were from Northern Italy. A team of ophthalmologists and medical geneticists (the latter from MAGI Human Medical Genetics Institute, Rovereto, Italy) evaluated all patients for diagnostic purposes, including which form of RP was to be confirmed at the molecular level. All subjects enrolled in the study underwent comprehensive ophthalmic examination, including slit-lamp examination, tonometry, and best-corrected visual acuity as evaluated using Early Treatment Diabetic Retinopathy Study charts and procedures. Goldmann kinetic perimetry (Goldmann Perimeter; Haag Streit AG; Mason, OH, USA) with a V4e target was performed on all patients. An I4e target was used to provide greater detail regarding the visual fields of patients. Fundus autofluorescence, infrared reflectance retinography, optical coherence tomography (Spectralis HRA-OCT; Heidelberg Engineering; Heidelberg, Germany), and electroretinograms following the guidelines of the International Society for Clinical Electrophysiology of Vision (MonPackOne; Metrovision; France), were performed in all patients. Demographic details, age of onset, and disease duration were recorded, along with all available information on personal and familial medical history, which was used to determine the type of genetic inheritance and to draw pedigrees.

The study followed institutional guidelines and the Declaration of Helsinki. Patients enrolled in the study signed 2 written informed consent forms, one for genetic testing and the other for publication of clinical and genetic data for research.

## Mutation screening

Peripheral blood samples obtained from patients and available family members were sent to the MAGI Laboratory (MAGI Human Medical Genetics Institute, Rovereto, Italy) for isolation of DNA and genetic testing. Genomic DNA was extracted using the Genra Puregene Blood Kit (Qiagen; Hilden, Germany) according to manufacturer instructions. DNA integrity and quality were evaluated by agarose gel electrophoresis and with a BioSpec-Nano spectrophotometer (Shimadzu Italia; Milan, Italy), respectively, prior to analysis.

Specific protocols for direct sequencing of each form of RP were previously optimized at the MAGI Laboratory. A total of 13 genes (Table 1) to be analyzed for different syndromic and non-syndromic forms of RP were selected based on mutation prevalence rates reported in the literature and on international guidelines (Pagon and Daiger, 2000; Waters and Beales, 2003; Fahim et al., 2013).

All coding regions and the intron/exon junctions of genes selected were amplified by polymerase chain reaction (PCR), purified, and analyzed using a Beckman Coulter CEQ 8000 sequencer (Beckman Coulter; Milano, Italy). PCR primer sequences and conditions are available upon request.

**Table 1.** Genes analyzed for each form of retinitis pigmentosa.

Gene	Reference sequence (GenBank)	Disease
<i>RHO</i>	NM_000539.3	adRP, sRP
<i>PRPH2</i>	NM_000322.4	adRP, sRP
<i>RP1</i>	NM_006269.1	adRP
<i>PRPF3</i>	NM_004698.1	adRP
<i>PRPF31</i>	NM_015629.3	adRP
<i>IMPDH1</i>	NM_000883.3	adRP
<i>RPE65</i>	NM_000329.2	arRP
<i>EYS</i>	NM_001142800.1	arRP, sRP
<i>RP2</i>	NM_006915.1	XL-RP
<i>RPGR</i>	NM_000328.2	XL-RP
<i>RPGR-ORF15</i>	NM_001034853.1	XL-RP
<i>USH2A</i>	NM_007123.4	USHER 2A
<i>BBS1</i>	NM_024649.4	BBS
<i>BBS10</i>	NM_024685.3	BBS

## Mutation and bioinformatic analysis

The electropherograms of all fragments amplified were analyzed using ChromasPro 1.5 (Technelysium Pty Ltd.; Australia) and Sequencher 5.0 (Gene Codes®; Ann Arbor, MI, USA) software and compared to GenBank reference sequences (Table 1) using the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov>). All mutations detected were sequenced bidirectionally at least twice for confirmation. DNA mutation nomenclature reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence.

Sequence variations that were ambiguously reported in the literature and/or in dbSNP (<http://www.ncbi.nlm.nih.gov/SNP/>) as benign polymorphisms were further analyzed for their putative pathogenicity. To identify mutations previously reported as pathogenic, the Human Gene Mutation Database (<http://www.hgmd.cf.ac.uk>) and Retina International database (<http://www.retina-international.org>) were consulted. Missense mutations were further investigated using MutationTaster (<http://www.mutationtaster.org>), Sorting Intolerant From Tolerant (<http://sift.jcvi.org/>), and Polyphen-2 (Polymorphism Phenotyping v2; <http://genetics.bwh.harvard.edu/pph2>) algorithms to predict their functional impact. Nonsense, frameshift, and canonical splice site variants were considered to be pathogenic.

All potentially pathogenic variants were detected by direct sequencing of 100 healthy subjects from the same geographic area. When available, DNA samples from family members were analyzed to confirm any cosegregation of the mutation with the phenotype.

## RESULTS

### Patients included in the study

Of the 56 unrelated probands enrolled in the study, 41 (73.2%) had non-syndromic RP and 15 (26.8%) had syndromic forms. Among patients with non-syndromic RP, 23 (56.1%) were familial cases and 18 (43.9%) were sporadic cases. Pedigree analysis of patients with inherited forms of the disease led to the classification of 9 adRP cases, 6 arRP cases, and 8 probands with XL-RP. Among patients with syndromic disease, 13 (86.7%) were classified as Usher2A and the remaining 2 (13.3%) were classified as having Bardet-Biedl syndrome.

### Mutation screening

Putative disease-causing mutations were identified in 19 of the 56 unrelated RP probands analyzed in this study (33.9% of cases). Mutation frequencies were 22.2% (2 mutation carriers in 9 patients; 2/9) for adRP; 16.7% for arRP (1/6) and sporadic patients (3/18), 50% for XL-RP (4/8) and patients with Bardet-Biedl syndrome (1/2), and 61.5% (8/13) for Usher2A patients.

We identified a total of 22 different heterozygous variants not previously reported in the literature and/or in dbSNP as benign polymorphisms, which were absent in the 100 control subjects. These included 10 missense mutations, 7 frameshift mutations, 2 nonsense mutations, 2 splicing mutations, and 1 variant in a regulatory region. Eight of 22 sequence variations were observed in the *USH2A* gene, 4 in the *RPGR* gene (3 of these in the ORF15 exon), 5 in *EYS*, 2 in *RHO*, 1 in the *PRPH2* gene, and 2 in the Bardet-Biedl-associated *BBS10* gene.

Twelve of these putative pathogenic mutations (54.5% of identified variants) are reported for the first time (Table 2), while 10 have been previously described in RP patients (Table 3). The new variants are located in *USH2A* (3 mutations), *RPGR* (3), *EYS* (5), and *RHO* (1).

The nonsense and the 3 frameshift variants were considered to be pathogenic. To predict the impact on protein function of the 7 missense mutations, we performed *in silico* analysis using 3 different algorithms. For 5 of these mutations (p.Gly182Val in *RHO*; p.Cys2139Arg and p.Cys2927Arg in *EYS*; p.Gly148Val in *RPGR*; p.Cys691Arg in *USH2A*; Table 2), at least 2 bioinformatic tools predicted the variants to be deleterious and the mutations were therefore considered to be pathogenic. The *USH2A* variant p.Asn1313Ser was evaluated as pathogenic

only by MutationTaster, while the p.Pro1631Ser missense variant in *EYS* was unequivocally predicted to be benign.

**Table 2.** Prediction of the functional impact of new mutations.

Proband	Phenotype	Gene	Nucleotide change	Protein effect	SIFT	Mutation Taster	PolyPhen2
P2 [F2]	adRP	<i>RHO</i>	c.545G>T	p.Gly182Val	P* [0]	P [0.99]	Prob <sup>#</sup> [1]
P15 [F3]	arRP	<i>EYS</i>	c.-459C>T	Unknown	-	-	-
	arRP	<i>EYS</i>	c.9299_9302del	p.Thr3100Lysfs*26	-	-	-
P24	sRP	<i>EYS</i>	c.4891C>T	p.Pro1631Ser	B <sup>†</sup> [0.06]	B [0.99]	B [0.12]
	sRP	<i>EYS</i>	c.6415T>C	p.Cys2139Arg	P [0]	B [0.99]	Prob [0.97]
P33	sRP	<i>EYS</i>	c.8779T>C	p.Cys2927Arg	P [0]	B [0.85]	Prob [0.99]
P35 [F5]	XL-RP	<i>RPGR-ORF15</i>	c.2023G>T	p.Glu675*	-	-	-
P36 [F6]	XL-RP	<i>RPGR</i>	c.443G>T	p.Gly148Val	P [0]	P [0]	Prob [0.99]
P39 [F11]	XL-RP	<i>RPGR-ORF15</i>	c.1967del	p.Asp656Valfs*41	-	-	-
P45	USHER2	<i>USH2A</i>	c.2071T>C	p.Cys691Arg	P [0]	P [0.99]	Prob [0.99]
P51	USHER2	<i>USH2A</i>	c.2013_2016del	p.Arg671Serfs*84	-	-	-
P52 [F10]	USHER2	<i>USH2A</i>	c.3938A>G	p.Asn1313Ser	B [0.42]	P [0.83]	B [0.03]

\*P = pathological; #Prob = probably pathological; B<sup>†</sup> = benign.

**Table 3.** List of known pathogenic mutations identified.

Proband	Phenotype	Gene	Nucleotide change	Protein effect	Reference <sup>§</sup>
P8 [F1]	adRP	<i>PRPH2</i>	c.493T>C	p.Cys165Arg	CM074464
P22	sRP	<i>RHO</i>	c.1040C>T	p.Pro347Leu	CM900199
P38 [F4]	XL-RP	<i>RPGR-ORF15</i>	c.2405_2406del	p.Glu802Glyfs*32	CD004115
P43	BBS	<i>BBS10</i>	c.1091del	p.Asn364Thrfs*5	White, 2007
	BBS	<i>BBS10</i>	c.1677del	p.Tyr559*	CD064494
P44 [F7]	USHER2	<i>USH2A</i>	c.2168-1G>C	-	CS073533
P49 [F8]	USHER2	<i>USH2A</i>	c.2299del	p.Glu767Serfs*21	CD982997
P50	USHER2	<i>USH2A</i>	c.187C>T	p.Arg63*	CM001804
	USHER2	<i>USH2A</i>	c.1663C>G	p.Leu555Val	CM014225
P55 [F9]	USHER2	<i>USH2A</i>	c.2167+5G>A	-	CS021381
P56	USHER2	<i>USH2A</i>	c.2299del	p.Glu767Serfs*21	CD982997

<sup>§</sup>Refers to HGMD accession number (Human Gene Mutation Database; <http://www.hgmd.cf.ac.uk>) or "Reference" section.

### New variants associated with non-syndromic RP

The age of patients with non-syndromic forms of RP ranged from 9-80 years (mean: 48 years), with a mean age at onset of 25 years. Molecular screening results were positive for 10 of 41 patients with clinical diagnosis of non-syndromic RP (24.4%). Clinical features and mutation details for probands whose causative mutations were identified are reported in Table 4. Nine of the 12 potential mutations (75%) identified in this screening have not been previously described.

#### RHO p.Gly182Val

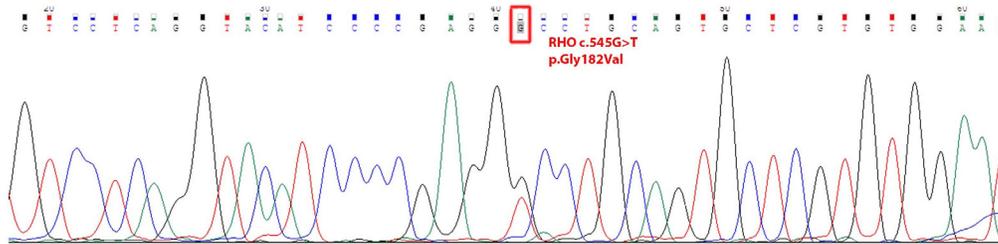
This mutation was detected as heterozygous in one adRP proband (P2-F2), a 40-year-old woman whose mother also had RP (Figure 1). The p.Gly182Val variant was a new missense mutation in exon 3 of the *RHO* gene (c.545G>T). The glycine residue affected was located in the extracellular domain of the protein and was found to be highly conserved in all organisms tested, suggesting that this amino acid has an important functional role (Figure 1).

**Table 4.** Clinical features of positive nonsyndromic retinitis pigmentosa patients.

Patient	Pathology	Mutation (Gene)	Age/Age of onset	Initial symptoms <sup>2</sup>	Visual acuity <sup>3</sup> (LogMAR)		Visual field <sup>4</sup>	Fundus appearance <sup>5</sup>	ERG <sup>6</sup>
					RE	LE			
P2	adRP	p.Gly182Val (RHO)	40/20	NB, VFD	0.8	0.8	MC	AA, SP	PSPH
P8	adRP	p.Cys165Arg (PRPH2)	56/50	NB	0.2	0.1	AS	AA, BS, SP	SR
P15	arRP	p.Thr3100Lysfs*26 (EYS) c.-459C>T (EYS)	60/45	BV	0.2	0.2	MC	POD, AA, BS, CME	PSPH
P22	sRP	p.Pro347Leu (RHO)	27/20	NB, VFD	0.0	0.04	MC	AA, BS	PSPH
P24	sRP	p.Pro1631Ser (EYS)	52/22	BV, NB, PP	0.15	0.5	T	POD, BS, AA, CME	Estingushed
P33	sRP	p.Cys2139Arg (EYS)	78/15	NB, VFD, BV	0.2	0.3	T	POD, AA, BS	Estingushed
P35	XL-RP	p.Glu675* (RPGR-ORF15)	45/5	NB	1.0	1.3	T	POD, AA, BS, MA	Estingushed
P36	XL-RP	p.Gly148Val (RPGR)	42/Asympt <sup>1</sup>	Asympt	0.0	0.0	MC	Sectoral BS	Normal
P38	XL-RP	p.Glu802Glyfs*32 (RPGR-ORF15)	55/20	NB, VFD	HM <sup>1</sup>	HM	ND	POD, AA, BS, MA	Estingushed
P39	XL-RP	p.Asp656Valfs*41 (RPGR-ORF15)	22/18	NB, VFD	0.3	0.3	T	AA, BS, MA	Estingushed

<sup>1</sup>Asympt = Asymptomatic. <sup>2</sup>NB = night blindness; VFD = visual field defect (contraction); BV = blurred vision; PP = photophobia. <sup>3</sup>HM = hand motion. <sup>4</sup>MC = mild contraction; AS = annular scotoma; T = tubular ≤ 10°; ND = not detectable. <sup>5</sup>AA = attenuated arterioles; BS = bone spicules; SP = salt/pepper; POD = pale optic disc; CME = cystoid macular edema; MA = macular atrophy. <sup>6</sup>PSPH = partial sparing of photopic ERG; SR = severe reduction of scotopic, maximal and photopic ERG.

Indeed, all software used for *in silico* analysis predicted that the substitution affected protein function (Table 2). A pathogenic missense mutation in the same codon was previously reported by Sheffield et al. (1991) (p.Gly182Ser).



Species	Match	Aa	Alignment
Human		182	F L A G W S R Y I P E <b>G</b> L Q C S C G I D Y Y T L
mutated	not conserved	182	E <b>V</b> L Q C S C G I D Y Y T
Ptrogodytes	all identical	182	E <b>G</b> L Q C S C G I D Y Y T
Mmulatta	all identical	182	E <b>G</b> L Q C S C G I D Y Y T
Foatus	all identical	182	E <b>G</b> M Q C S C G I D Y Y T
Mmusculus	all identical	182	E <b>G</b> M Q C S C G I D Y Y T
Ggallus	all identical	104	F L F G W S R Y I P E <b>G</b> M Q C S C G I D Y Y T
Trubripes	all identical	182	E <b>G</b> M Q C S C G V D Y Y T
Drexio	all identical	182	E <b>G</b> M Q C S C G V D Y Y T
Dmelanogaster	all identical	182	E <b>G</b> M Q C S C G V D Y Y T
Celegans	no homologue		
Xtropicalis	all identical	202	F L I G W S A Y V P E <b>G</b> N L T A C S I D Y M T

**Figure 1.** Sequence chromatogram of the p.Gly182Val mutation in *RHO* exon 3 identified in autosomal dominant RP patient P2. Conservation of affected amino acids across species determined by the MutationTaster tool is shown below.

### ***EYS* c.-459C>T and p.Thr3100Lysfs\*26**

In proband P15 of family F3, a 60-year-old woman, molecular screening for genes involved in arRP (Table 1) revealed 2 different heterozygous variants in the *EYS* gene: a mutation in non-coding exon 1 (c.-459C>T) and a small deletion (c.9299\_9302del; p.Thr3100Lysfs\*26). Although the effect of the first variant on protein function could not be evaluated using bioinformatic tools, it is likely to be pathogenic as 3 different genetic alterations in the same region of *EYS* have been described (Barragán et al., 2010). Their potential pathogenicity is thought to be related to their location in regulatory regions that are important for protein translation (Scheper et al., 2007). The second new variant detected in P15 was considered to be pathogenic because deletion of 4 nucleotides at codon 3100 in exon 43 led to a frameshift and premature protein termination (p.Thr3100Lysfs\*26).

### ***EYS* p.Pro1631Ser and p.Cys2139Arg**

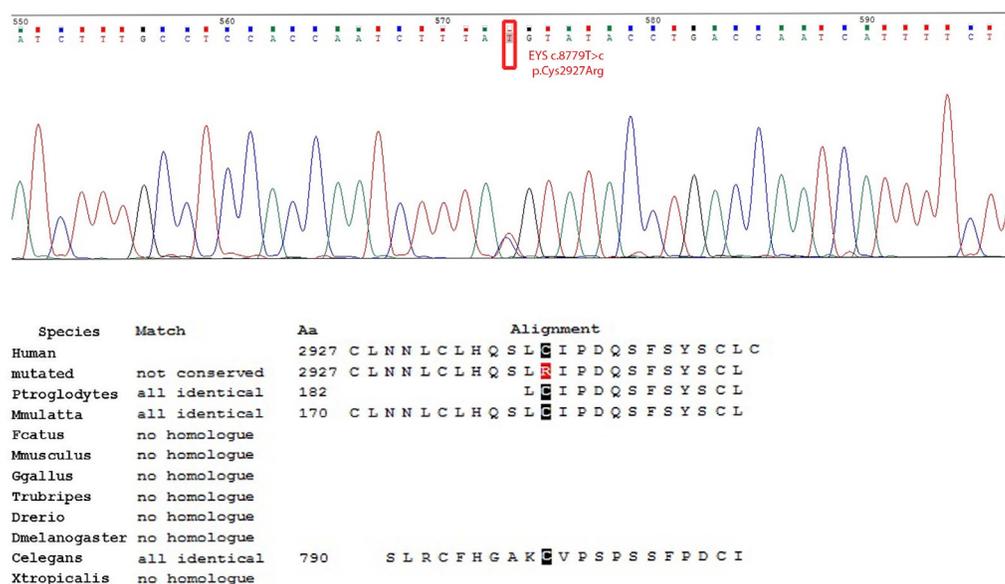
The proband with these 2 missense variations (P24) was a 52-year-old woman. Since

no other members affected were identified in the family, this case was presumed to be a sporadic case. Both putative mutations (c.4891C>T, p.Pro1631Ser; c.6415T>C, p.Cys2139Arg) were identified as heterozygotes in the *EYS* gene. Although the p.Pro1631Ser variant in exon 27 has not been reported as a polymorphism in the literature or databases and was not identified in our control samples, all prediction tools indicated it to be benign. Notably, the same variant was detected in the unaffected son of P24. Further analyses are required to clarify the potential role of this genetic variant.

The missense mutation, p.Cys2139Arg, in exon 32 causes substitution of the polar residue cysteine 2139 with the basic arginine in the epidermal growth factor (EGF)-like 21 domain. Since Cys2139 is conserved in primates but not in other species, the effect of its substitution on protein function was determined to be benign by the MutationTaster tool, while Sorting Intolerant From Tolerant and Polyphen-2 predicted the variants to be deleterious. A pathogenic missense mutation in the same codon (p.Cys2139Tyr) was described by Audo et al. (2010), thus supporting the pathogenic potential of the variant detected in P24.

### *EYS* p.Cys2927Arg

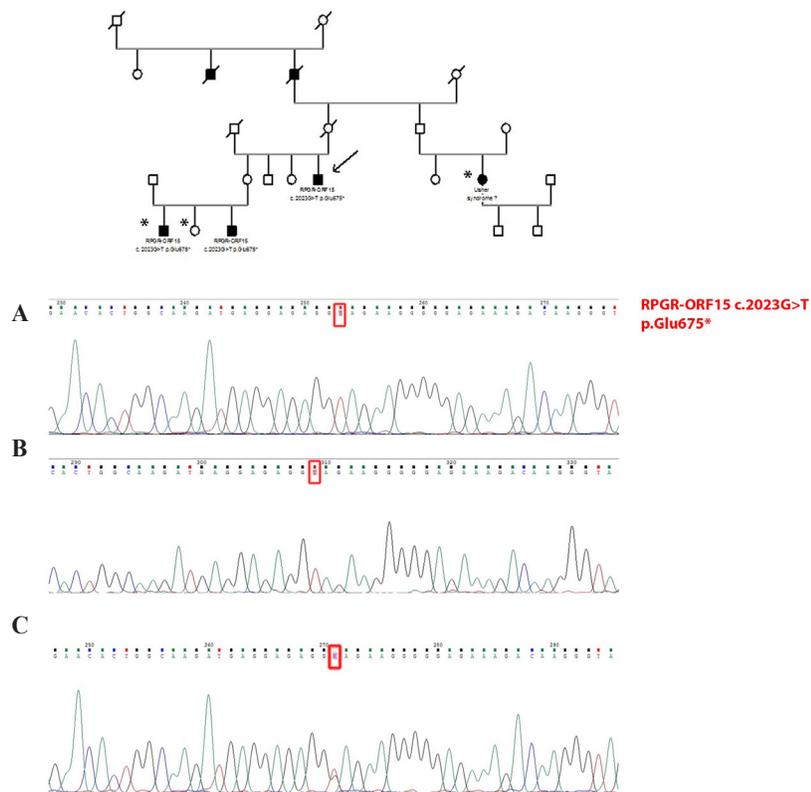
This missense mutation (c.8779T>C; Figure 2) was observed as a heterozygous mutation in one 78-year-old male patient (P33). The c.8779T>C variant was located on exon 43 in the EGF-like 26 domain and resulted in a substitution of a cysteine residue that is conserved in primates and in *Caenorhabditis elegans* (Figure 2). Two of the 3 bioinformatic tools predicted the variant to be deleterious, and it was therefore classified as potentially pathogenic.



**Figure 2.** Sequence chromatogram of the heterozygous p.Cys2927Arg mutation in *EYS* identified in sporadic retinitis pigmentosa proband P33. This variant was on exon 43 in the EGF-like 26 domain and resulted in substitution of a cysteine residue that is conserved in primates and in *Caenorhabditis elegans*.

**RPGR p.Glu675\***

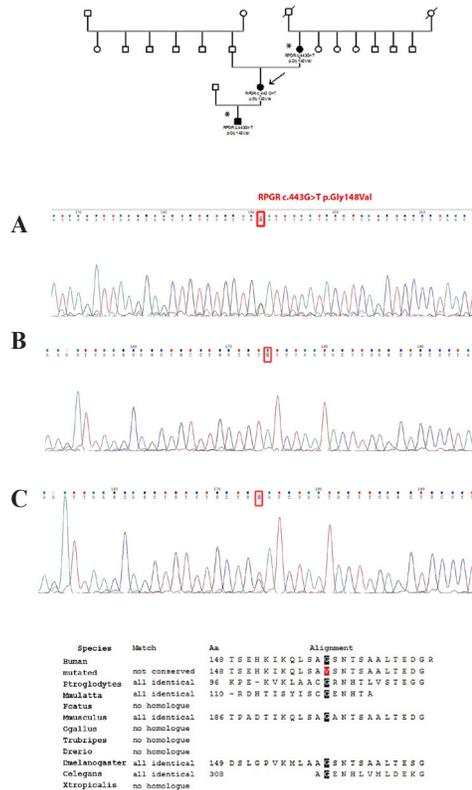
This new nonsense mutation was identified in family F5, which contains 6 members with RP. Pedigree analysis suggested X-linked inheritance (Figure 3). In the proband (P35), a 45-year-old male, molecular screening identified the hemizygous nonsense variant c.2023G>T on the ORF15 exon of the *RPGR* gene, which results in the synthesis of a truncated protein (p.Glu675\*) (Figure 3). In this family, the mutation segregated with the disease phenotype. Indeed, the same genetic alteration was confirmed in the affected nephew and niece of the proband (Figure 3). The proband's nephew, a 17-year-old male, first showed symptoms of night blindness at 11 years of age. The niece's proband, a 20-year-old female, had severe myopia (-8.00 sphere in both eyes), with good central visual acuity (0.0 logMAR in both eyes) and atypical retinal pigmentation adjacent to the vascular arcade. The p.Glu675\* mutation was not identified in the first cousin of P35, who showed clinical signs of RP starting at 24 years of age and neurosensory hearing deficit at birth; Usher syndrome was therefore postulated for this patient.



**Figure 3.** Pedigree of family F5 suggesting X-linked inheritance of retinitis pigmentosa. Molecular screening of proband P35 identified the new hemizygous nonsense variant p.Glu675\* (c.2023G>T) on the ORF15 exon of the *RPGR* gene (A). The same genetic alteration was confirmed in the affected nephew (B) and niece (C) of the proband. Solid symbols indicate affected individuals. Asterisks indicate family members for whom DNA was available for genomic analysis; arrow indicates the proband.

**RPGR p.Gly148Val**

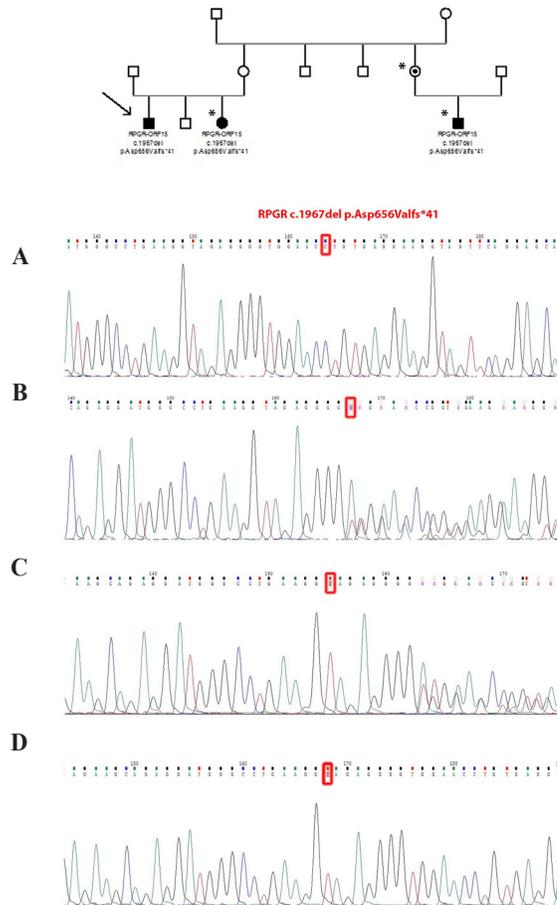
This new mutation was identified as a heterozygous variant in proband P36, a 42-year-old female whose mother and son also had RP (Figure 4). She was asymptomatic and had not noticed any changes in her visual field, although Goldmann perimetry showed superotemporal scotoma in the left eye. Fundus examination revealed that the retina had a salt-and-pepper pattern and bone-spicule-shaped pigment changes in the inferior periphery. Electroretinography testing was normal in both eyes, with lower amplitudes in the left eye. Monolateral visual field loss corresponding to fundus abnormalities observed in P36 confirmed the clinical data of mildly affected X-linked recessive RP carriers showing phenotypic signs of the disease, depending on non-random X chromosome inactivation (Koenekoop et al., 2003). The son of P36, who was 12 years old, presented with a history of night blindness at 5 years old. The rapid progression of RP observed in this young patient indicates the severity of XL-RP in affected males, which often leads to legal blindness by the end of the 4th decade. In this family, the c.443G>T mutation segregated with the RP phenotype and was detected in the mother and son of the patient P36 (Figure 4), confirming that this variant is disease-causing. This was also indicated by bioinformatic prediction of the mutation impact.



**Figure 4.** Pedigree of family F6 with X-linked inheritance of retinitis pigmentosa (RP). The new p.Gly148Val mutation was identified in *RPGR* exon 5 as a heterozygous variant in proband P36 (chromatogram A), a 42-year-old female whose son and mother also had RP (chromatograms B and C, respectively). Solid symbols indicate affected individuals. Asterisks indicate family members for whom DNA was available for genomic analysis; arrow indicates the proband.

**RPGR p.Asp656Valfs\*41**

There were several members affected in family F11 of proband P39 (Figure 5). The proband (P39), a 22-year-old male, had night blindness for as long as he could remember, with peripheral vision problems beginning at age 14. Molecular screening for XL-RP-associated genes revealed a new frameshift variant on the ORF15 exon of the *RPGR* gene (c.1967del). This variant resulted in synthesis of a truncated protein and was therefore considered to be pathogenic. Sequencing analysis confirmed the same variant in the affected sister (heterozygous mutation), aunt (heterozygous mutation), and cousin (hemizygous mutation) of the proband, indicating X-linked inheritance in this family.



**Figure 5.** Pedigree of family F11. Molecular screening of patient P39 for XL-RP-associated genes revealed a new frameshift variant on the ORF15 exon of the *RPGR* gene (c.1967del; chromatogram A). Sequencing analysis confirmed the same variant in the affected sister (heterozygous mutation; chromatogram B), aunt (heterozygous mutation; chromatogram C), and cousin (hemizygous mutation; chromatogram D) of the proband. Solid symbols indicate affected individuals. Asterisks indicate family members for whom DNA was available for genomic analysis; arrow indicates the proband.

### **New variants associated with syndromic RP**

The age of patients affected by syndromic forms of RP ranged from 21-79 years (mean age: 45 years), with a mean age at onset of 16 years (range 0-31 years). Molecular screening was positive for 9 of 15 patients with clinical diagnosis of Usher2A or Bardet-Biedl syndrome (60% of cases). Clinical features and mutation details of probands with identified causative mutations are reported in Table 5. Three of the 8 potential mutations (37.5%) identified in this screening have not been previously reported. All of these new variants were on the *USH2A* gene (Table 2).

#### ***USH2A p.Cys691Arg***

This new mutation was detected in proband P45, a 30-year-old female patient whose familial medical history could not be ascertained. Molecular screening of the *USH2A* gene detected a new heterozygous variant on exon 12 (c.2071T>C; p.Cys691Arg) (Figure 6). This mutation causes the substitution of a highly conserved cysteine residue with a basic arginine at codon 691 in the extracellular laminin EGF-like 3 domain of the protein. The importance of this residue is further supported by the results obtained through *in silico* analysis using 3 different algorithms, which univocally indicated this mutation to be pathogenic. A missense pathogenic mutation affecting the same codon has also been described (Nakanishi et al., 2009).

#### ***USH2A p.Arg671Serfs\*84***

A new frameshift mutation involving deletion of a base on exon 12 (c.2013\_2016del) was identified in proband P51, a 79-year-old woman who noted neurosensory hearing impairment at the age of 6 years with night blindness and loss of peripheral vision at 18 years. This heterozygous variant led to synthesis of a truncated protein (p.Arg671Serfs\*84) and was found to be located in the extracellular laminin EGF-like 3 domain of the protein, similarly to the new variant identified in P45.

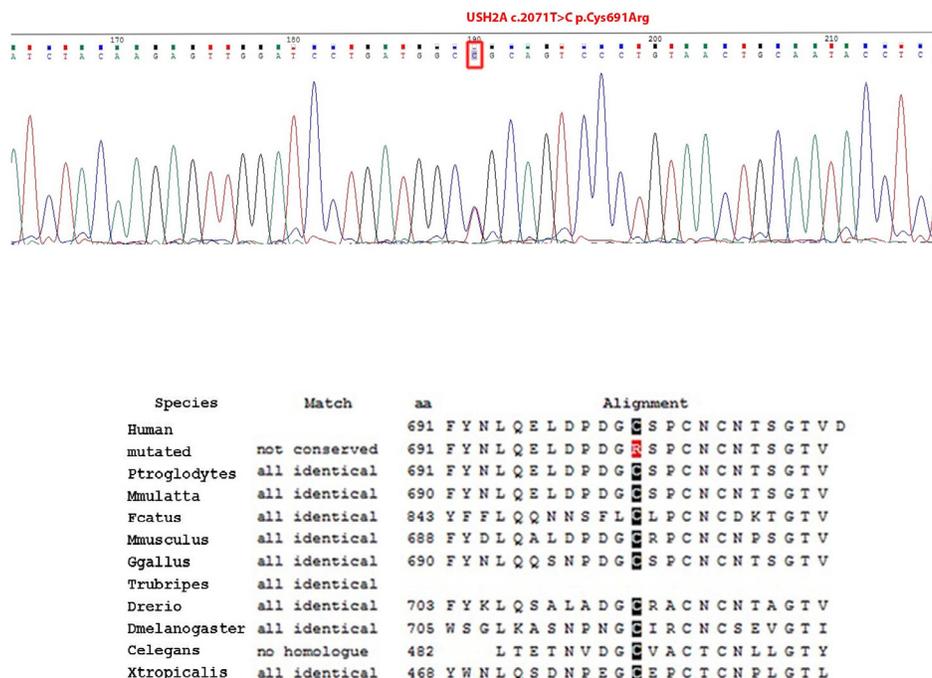
#### ***USH2A p.Asn1313Ser***

A new missense mutation (c.3938A>G) located on exon 18 of the *USH2A* gene was found to be heterozygous in proband P52, a 56-year-old woman with a family history of Usher syndrome. She presented with a 6-year history of neurosensory hearing impairment and reported dark adaptation problems and peripheral visual field problems since the age of 14 years. The c.3938A>G variation led to substitution of a polar asparagine residue with a serine residue at position 1313 in the extracellular fibronectin type-III 3 domain of the usherin protein. Two prediction tools indicated low pathogenic potential for this amino acid change, while MutationTaster predicted an impact on protein function, as this position is highly conserved in various organisms.

**Table 5.** Clinical features of positive syndromic retinitis pigmentosa patients.

Patient	Pathology	Mutation (Gene)	Age/Age of onset	Initial symptoms <sup>2</sup>	Visual acuity <sup>3</sup> (LogMAR)		Visual field <sup>4</sup>	Fundus appearance <sup>5</sup>	ERG
					RE	LE			
P43	BBS	p.Asn364Thrfs*5 ( <i>BBS10</i> )	32/5	NB	LP <sup>3</sup>	ND	POD, AA, BS, MA	Extinguished	
P44	USH2R2	p.Tyr559* ( <i>BBS10</i> )	44/22	NB	1.3	ND	POD, AA, BS, MA	Extinguished	
P45	USH2R2	c.2168-1C>C ( <i>USH2A</i> )	30/8	NB	0.0	MC	POD, AA, BS, CME	Extinguished	
P49	USH2R2	p.Cys691Arg ( <i>USH2A</i> )	62/14	NB, VFD	0.2	T	POD, AA, BS	Extinguished	
P50	USH2R2	p.Glu767Serfs*21 ( <i>USH2A</i> )	50/28	BV	0.5	T	POD, AA, BS	Extinguished	
P51	USH2R2	p.Arg63* ( <i>USH2A</i> )	79/18	NB	1.7	ND	POD, AA, BS, MA	Extinguished	
P52	USH2R2	p.Arg671Serfs*84 ( <i>USH2A</i> )	56/14	PP, VFD	1.3	T	POD, AA, BS	Extinguished	
P55	USH2R2	p.Asm1313Ser ( <i>USH2A</i> )	65/30	PP, VFD	LP	ND	POD, AA, BS, MA	Extinguished	
P56	USH2R2	c.2167+5G>A ( <i>USH2A</i> )	NA <sup>1</sup>	NA	NA	NA	NA	Extinguished	

<sup>1</sup>NA = not available; <sup>2</sup>NB = night blindness; VFD = visual field defect (contraction); BV = blurred vision; PP = photophobia; <sup>3</sup>LP = light perception; <sup>4</sup>MC = mild contraction; T = tubular  $\leq 10^\circ$ ; ND = not detectable; <sup>5</sup>POD = pale optic disc; AA = attenuated arterioles; BS = bone spicules; CME = cystoid macular edema; MA = macular atrophy.



**Figure 6.** Sequence chromatogram of the new mutation p.Cys691Arg in *USH2A* detected in proband P45, a 30-year-old female whose familial medical history could not be ascertained. This position has been highly conserved throughout evolution, as determined using the MutationTaster tool.

## DISCUSSION

In the present study, collaboration between a referral ophthalmology department and a team of geneticists with knowledge of retinal dystrophies enabled clinical and genetic characterization of an Italian case series of 56 probands with different forms of non-syndromic and syndromic RP. We enrolled all patients clinically suspected of having RP without any other selection criterion; therefore, our sample included affected subjects who report annually to our University Eye Clinic and are offered multidisciplinary counseling.

This is the first Italian study to analyze non-syndromic and syndromic RP patients, including familial and sporadic cases. There have been only 3 studies examining the molecular epidemiology of specific forms of RP in Italy: a study on 43 adRP families (Ziviello et al., 2005), a characterization of 75 families with Usher syndrome (Vozzi et al., 2011), and a report on 15 familial cases of XL-RP (Miano et al., 1999).

We carried out mutation analysis in 41 index patients with non-syndromic RP and different inheritance patterns and 15 probands with syndromic RP. We identified causative mutations in 19 of the 56 probands (33.9%), detecting 22 different heterozygous mutations, 12 of which are new variants not previously reported.

In patients with non-syndromic RP, molecular screening was positive for 10 of 41 probands (24.4%). The results for adRP cases showed similar test positivity to that (27.9%) reported by Ziviello et al. (2005); however, some of the genes analyzed in their panel were

different from those analyzed in our study (including *NRL*, *CRX*, and *PRPF8*). A causative variant was found in 2 of 9 adRP patients (22.2%). As discussed above, the p.Cys165Arg mutation in the *PRPH2* gene, previously reported by Simonelli et al. (2007), and the new variant detected in the *RHO* gene (p.Gly182Val) were classified as pathogenic.

Molecular screening of 6 arRP probands and 18 sporadic cases led to the identification of heterozygous variants in the *EYS* and *RHO* genes. Five new sequence variants in the *EYS* gene were detected in our screening. As described above, 4 of these novel variants (p.Thr3100Lysfs\*26, c.-459C>T, p.Cys2139Arg, and p.Cys2927Arg) were classified as pathogenic, while the p.Pro1631Ser variant identified in sporadic proband P24 was classified as neutral based on bioinformatic analysis. Functional studies will be performed to assess the effect of all newly identified mutations on protein function.

The p.Pro347Leu missense mutation in *RHO*, which we detected in a sporadic RP patient initially showing RP symptoms at the age of 20 years, was previously associated with adRP in the Italian population (Dryja et al., 1990; Ziviello et al., 2005). The association of this mutation with early onset of disease at 12-23 years of age (Ziviello et al., 2005; Simonelli et al., 2007; Kim et al., 2011) was confirmed in our study.

In agreement with a previous study, we found that *EYS* was mainly associated with sporadic and recessive forms of non-syndromic RP (Abd El-Aziz et al., 2008). Notably, this is the first study identifying the association between *EYS* mutations and non-syndromic RP forms in the Italian population.

Genetic test positivity was significant in index patients with XL-RP who were analyzed for *RP2* and *RPGR* point mutations (4 of 8 positive cases); we also screened the ORF15 region of *RPGR*, a unique terminal exon present in the alternatively spliced transcript, which is the predominant form of *RPGR* in the retina (Vervoort et al., 2000; Pusch et al., 2002; Sharon et al., 2003). Three *RPGR* mutations were detected in ORF15 (p.Glu802Glyfs\*32, p.Glu675\*, and p.Asp656Valfs\*41) and 1 was found in *RPGR* exon 5 (p.Gly148Val). The latter variant and the 2 frameshift mutations affecting *RPGR*-ORF15 were confirmed to segregate with the disease phenotype in other family members available for genetic testing. As expected, patients with XL-RP due to mutation in the *RPGR* gene were found to have a severe form of inherited retinal degeneration, characterized by childhood onset, rapid progression, and early macular involvement with decreased visual acuity, leading to significant vision loss before the 4th decade (P35); various degrees of retinal dysfunction were described in heterozygous carrier females.

A previous study examined 15 Italian families with XL-RP (Miano et al., 1999), but the ORF15 region, which was identified in a later study (Vervoort et al., 2000), was not screened. We therefore report the results of molecular screening of the ORF15 exon in Italian families for the first time. Our data agrees with the results of international studies indicating that *RPGR*-ORF15 mutations are responsible for 60-80% of XL-RP cases (Vervoort et al., 2000).

Genetic test positivity for non-syndromic RP patients (24.4%) detected in the present study agreed with the results of previous studies. The significant proportion of negative test results may have been observed because of the high clinical and genetic heterogeneity of RP, which is reflected by the number of underlying gene defects. Moreover, the prioritization of genes for analysis by Sanger sequencing is limited by the lack of a specific correlation between particular ophthalmological characteristics and genetic subtypes of RP, and molecular diagnostics is particularly challenging for examining isolated RP patients (Nájera et al., 1995; Hayakawa et al., 1997).

Syndromic forms of RP, such as Usher2A and Bardet-Biedl syndrome, show some differences; the disease-causing genes identified thus far have been associated with an increased number of cases (*USH2A* with approximately 80% of patients with Usher syndrome type II; *BBS1* and *BBS10* with approximately 50% of Bardet-Biedl cases).

We identified causative mutations, including 2 known frameshift variants in the *BBS10* gene, in 1 of 2 probands with Bardet-Biedl syndrome. Direct sequencing of exons 2-21 of the *USH2A* gene revealed heterozygous variants in 8 of 13 patients (61.5%); the 2 causative mutations, expected to be associated with this recessive disorder, were only detected in 1 proband (P50). This is the first phase our screening, which did not include direct sequencing of exons 22-72 or detection of gross deletions. This protocol is based on previous studies (Aller et al., 2010; McGee et al., 2010). Analysis of the longer isoform of *USH2A* clearly increases the detection rate, and a second screening step is currently underway to complete the genetic testing of *USH2A* patients. We detected a total of 8 different mutations, 3 of which are new variants. A similar study performed in 39 Italian patients, using a chip for the most frequent variants identified in Caucasians, revealed *USH2A* causative mutations in 15.4% of cases (Vozzi et al., 2011). Among the mutations already associated with Usher type II, the c.2299del variant was found in 25% of patients (2 of 8); this variant is the most common mutation detected in Europe and the USA and may have a South-European origin (Eudy et al., 1998; Aller et al., 2010). The c.2299del frameshift variant (p.Glu767Serfs\*21) and the nonsense mutation c.187C>T (p.Arg63\*) identified in our patients were found to be the most frequently observed mutations in the Italian population and are described in the present study and in Vozzi et al. (2011).

In summary, our results help define the spectrum and frequency of mutations associated with different forms of syndromic and non-syndromic RP in Italian patients. Notably, 12 of the 22 putative causative variants identified were new mutations that have not been reported previously. Since confirmation of new mutations in other family members was not always possible, analysis of additional families and proper functional studies are needed to confirm the pathogenicity of some of these variants.

This is the first report in Italy including sporadic patients and probands with arRP and the association between *EYS* mutations and non-syndromic RP forms. The study also provides data regarding *RPGR*-ORF15 mutations in XL-RP patients, and thus important information about mutations causing RP in the Italian population. Understanding the unique pattern of frequent mutations in a specific ethnic group may facilitate the development of molecular diagnosis and personalized gene therapies, which have recently made considerable advances (Liu et al., 2011; Beltran et al., 2012), as well as facilitate the counseling of patients.

The percentage of RP mutations identified in the present and previous Italian studies, along with difficulties in the prioritization of genes for Sanger sequencing, suggest that innovative methodology such as next-generation sequencing (NGS) will be necessary for significantly improving the molecular diagnosis of RP. Applications of NGS for diagnosing heterogeneous disorders such as RP are supported by recent studies (Audo et al., 2012; O'Sullivan et al., 2012).

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