

Specialist Information

from the Committee for Genetics and

Laboratory Animal Breeding

Substrains of Inbred Strains

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1 Introduction

An inbred strain is *per definitionem* generated, in that a strict consecutive Brother-Sister-Inbreeding (BSI) system is maintained over a period of 20 generations (F20). The BSI system continually increases inbreeding coefficient, i.e. the probability of a single gene locus becoming homozygous. As shown in Fig.1, a predicted value of 98.63 % is expected at generation F20. Since inbred strains continue to be bred by BSI after having reached the F20 generation, the inbreeding coefficient further increases during strain propagation. In generation F40 a value of 99.98 % is reached (see Fig. 1); at this point the residual heterozygosity can virtually be disregarded.

Importantly the residual heterozygosity between F20 and F40 as well as spontaneous mutations and accidental contaminations induce a genetic drift in inbred strains, which leads to the formation of substrains.

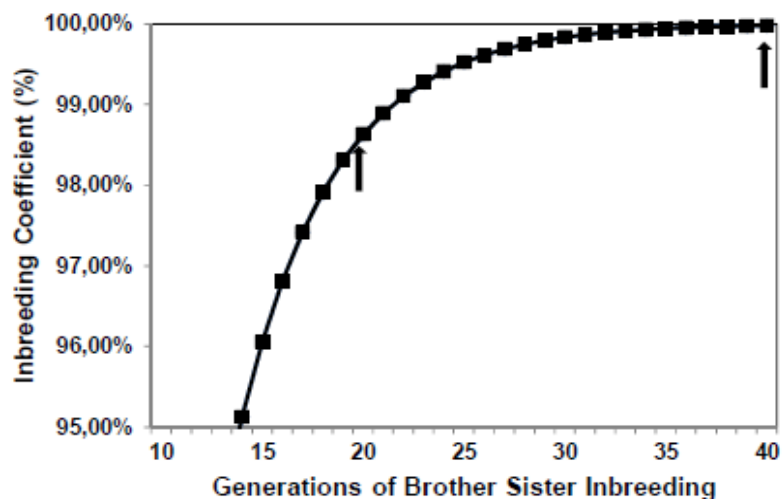


Figure 1: Inbreeding coefficient of an inbred strain in the course of a BSI breeding system. The inbreeding coefficient rises to a magnitude of 98.63 % up to F20 (time point at which a defined inbred strain is established, left arrow) and reaches a value of 99.98 % by F40 (right arrow). Consequently, residual heterozygosity between F20 and F40 is relatively high. Calculation for inbreeding coefficient resulted from the formula $F_t = 0.25 (1 + 2F_{t-1} + F_{t-2})$; Falconer 1989) and a value of 0.25 was applied for F1.

2 Definition of a substrain

A substrain is a branch or a subline of the original inbred strain, which exhibits genetic differences to the original colony of the inbred strain, which are either scientifically proven or exist with high probability. In the following the specific criteria are mentioned, which according to the current *Guidelines for Nomenclature of Mouse and Rat Strains* (<http://www.informatics.jax.org/mgihome/nomen/strains.shtml#nois>) lead to the obligation to postulate a new substrain.

2.1 Separation between F20 and F40

„If two branches are separated after 20 but before 40 generations of inbreeding there still will be enough residual heterozygosity that two genetically different substrains will result” (<http://www.informatics.jax.org/mgihome/nomen/strains.shtml#nois>). Because of the high proportion of residual heterozygosity to be expected between generations F20 and F40 (see Fig. 1) the separation of the subline from the original inbred colony in this interval of generations will in all probability lead to genetic variation.

2.2 Separation for 20 BSI generations after F40

„If branches are separated for more than 20 generations from a common ancestor, it is likely that genetic variation between the branches will have occurred by mutation and genetic drift (<http://www.informatics.jax.org/mgihome/nomen/strains.shtml#nois>). In this context 20 generations of separation is the sum of breeding generations required for the original colony and the substrain. Hence, the subline only needs to be bred separately from the original colony for approximately 10 generations to be classified as an independent substrain. The reason for postulation of a new substrain after 20 generations of breeding is because only minor residual heterozygosity is present after the F40 generation (see Fig. 1). After the F40 generation, genetic drift between the original colony and the respective subline is primarily mediated by spontaneous mutations (Radulovic et al. 1998; Sluyter et al. 1999; Stiedl et al. 1999; Specht und Schoepfer 2001; Roth et al. 2002; Wotjak 2003).

2.3 Genetic differences

„If genetic differences are proven by genetic analysis to have occurred between branches” (<http://www.informatics.jax.org/mgihome/nomen/strains.shtml#nois>).

3 Origin of substrains

As a rule substrains are not purposefully selected, they usually emerge by accident, inadvertently or in an unrecognized way.

At present many inbred strains are in existence that have already passed hundreds of generations and have frequently been separated during their history in order to provide the strain to other laboratories for establishing colonies of their own. Correspondingly the mechanisms specified under 2.1 and 2.2 have led to the frequent development of substrains. The Jackson Lab has published the impressive substrain diversity which developed in the C57BL/6 strain over the course of time <http://jackson.jax.org/rs/444-BUH-304/images/Genetic-Drift-Webinar-11May2017.pdf>

In the past substrains also emerged for other reasons, such as unintentional genetic contamination which occurred for example by incorrect mating's (Naggert et al. 1995), or inadequately documented and forgotten outbred stocks which had been established for specific scientific purposes (Bailey 1977; Bailey 1982; Simpson et al., 1997; Threadgill et al., 1997a; Threadgill et al. 1997b; Wotjak 2003).

New substrains are frequently recognized due to variations in their phenotype. In particular variations between substrains have often been detected by immunologists since they frequently work with very sensitive systems which respond to the slightest genetic variation of the experimental animals used (Bailey 1982). Furthermore, the reactions of different animal populations of an inbred strain to behavioural tests represent a good indicator for the emergence of substrains (Crawley et al., 1997; Crawley und Paylor, 1997, Stiedl et al. 1999). Further, histocompatibility tests (Simpson et al. 1997), tumor susceptibility (Glant et al. 2001), divergence of fear behaviour (Radulovic et al. 1998; Stiedl et al. 1999), differing physiological reactions to anesthetics (Roth et al. 2002) and varying thresholds for inducing epileptic seizures by electro impulse (Yang et al. 2003) have been utilized for discrimination of substrains in the past.

4 Nomenclature of substrains

A new substrain receives as a supplement to its strain name an additional laboratory code, which is specifically assigned to the institute in which the substrain was developed. This laboratory code consists of 1 - 5 characters which identify the institute, the laboratory or the scientist who has generated the strain or continues to breed it. For example, the code "J" stands for *The Jackson Laboratory*; the code "N" for the *National Institute of Health* or the code "CrI" for the *Charles River Laboratories*. The laboratory codes are allocated by the *Institute for Laboratory Animal Research* (ILAR, <http://dels.nas.edu/global/ilar/Lab-Codes>). To designate a substrain a slash is inserted directly after the strain name and the laboratory code is added (for example C57BL/6J and C57BL/6N). If the holder of the strain changes the new laboratory code is added without the use of the "slash" (for example C57BL/6NCrI, C57BL/6JHanZtm). Therefore, knowledge of the laboratory code can already provide a small part of the strain history. In accordance with the *Guidelines of the International Committee on Genetically Standardized Nomenclature for Mice* commercial suppliers should provide detailed information on the history of a strain in their internet presentations. For example, the Charles River Laboratory provides the following information with regard to the C57BL/6NCrI strain: "*Developed by C.C. Little in 1921, from a mating of Miss Abby Lathrop's stock that also gave rise to strains C57BR and C57L. Strains 6 and 10 separated about 1937. To The Jackson Laboratory in 1948 from Hall. To NIH in 1951 from The Jackson Laboratory at F32. To Charles River in 1974 from NIH.*" In 1975 the strain was re-derived by hysterectomy."

5 Discrimination of substrains

Normally substrains of an inbred strain have the same coat color, and therefore it is often difficult to distinguish them by their outward appearance. In contrast the genetic differences between substrains of the same inbred strain can be significant (see Table 2). Skin transplantations represent a relatively simple method which can be used to distinguish differences between substrains without excessive laboratory investment and expenditure. However, this technology is very time consuming and depends on well-trained personnel. Skin transplantations from the inbred strain C57BL/6J to C57BL/6N

for example lead to transplant rejection (see Recommendation from the *Committee for Genetics and Laboratory Animal Breeding* of GV-SOLAS „Zielsetzungen und Methoden des genetischen Monitoring isogener Maus- und Rattenstämme“).

Differentiation of substrains by molecular genetic methods is faster and can dependent on experience and laboratory infrastructure available be processed single handed, or alternatively be assigned to a commercial company. The molecular genetic markers used for this purpose are allele specific oligonucleotides (ASO), microsatellites (simple tandem repeats - STR) or single nucleotide polymorphisms (SNP). ASOs can be developed and employed if the original inbred strain and the substrain differ with respect to an already identified specific mutation. For example this applies to the mitochondrial *Nnt*-deficiency allele of the C57BL/6J strain (allele annotation: nicotinamide nucleotide transhydrogenase, C57BL/6J), or to the deletion of the *Snca* (alpha-synuclein) locus on chromosome 6 of a C57BL/6 sub-population which was distributed by Harlan (Specht und Schoepfer, 2001; Specht und Schoepfer, 2004; Huang et al., 2006; Aston-Mourny et al., 2007; Kim et al., 2010). In contrast microsatellites represent short repetitive base sequences that range from 100 to 1500 base pairs and are homogenously distributed in the genome. They can easily be detected by using PCR techniques and subsequent gel electrophoresis. SNPs are characterized by single nucleotide exchanges of the DNA strand. SNP polymorphisms are significantly more frequent than microsatellite markers. SNPs can be detected by sequencing techniques, special PCR methods, real time PCR or micro-array techniques.

The molecular-genetic profiles of most mouse and rat inbred strains have been published in standard data bases like *Mouse Genome Informatics* – MGI (<http://www.informatics.jax.org/>), *Rat Genome Database* – RGD (<http://rgd.mcw.edu>) or Ensembl (<http://www.ensembl.org/index.html>). Microsatellites suitable for differentiation of C57BL/6 substrains have been published; Hovland and colleagues (Hovland et al. 2000) only found 13 of 823 markers investigated to be informative for differentiation between the C57BL/6J and C57BL/6N substrains. Table 1 displays SNP polymorphisms which can be used to discriminate between the J and N substrains of the C57BL/6 strain.

Table 1: SNP polymorphisms between the C57BL/6N and C57BL/6J substrains (courtesy of *The Jackson Laboratory*)

SNP designation	genotypes	
	C57BL/6N	C57BL/6J
08-015199792-M	C	T
11-004367508-M	A	G
13-041017317-M	C	T
15-057561875-M	G	A
19-049914266-M	T	G

At the *Institute for Laboratory Animal Science and Central Animal Laboratory* of the Hannover Medical School, a panel of SNPs (n=39) has been developed, which can differentiate all C57BL/6 substrains that are currently available on the European market. This SNP panel is planned to be published in the near future.

In 2009 Mekada and co-workers (Mekada et al., 2009) published a panel of 12 SNPs which can differentiate all Nnt deficient B6 substrains.

Another method for molecular-genetic differentiation of substrains is identification of copy number variations (CNV). CNVs represent rather long DNA sequences which can be found in different copy numbers in inbred strains and their substrains. Very little research has been done with CNVs. However, CNV DNA elements are considered to play a significant role in phenotype formation since they can contain one or several genes (Cutler et al. 2007; Watkins-Chow and Pavan, 2008). If applicable, CNVs can be analyzed by sequencing, PCR or special hybridization techniques.

Table 2 displays the most important substrains of frequently used mouse inbred strains including their genetic and phenotypic specialties. The phenotype differences are -in part- well described and known for a long time. New research studies continuously bring to light further diversities between substrains which are particularly relevant for the B6 substrains since they are frequently used as the genetic background for genetically modified loci.

Table 2: Phenotypic and genetic differences between substrains of common mouse inbred strains

Inbred strain	Substrains	Phenotype	Genetic locus affected	Reference
AKR				
	AKR/Cu vs AKR/J	lymphoma cells of AKR/J donors are rejected by AKR/Cu recipients	MiHC	Acton et al., 1973 Zatz, 1978
BALB/c				
	BALB/cJ	70% incidence for pristane induced arthritis		
	BALB/cAn	20% incidence for pristane induced arthritis		
	BALB/cByJ	drinking and eating disorders (less fat consumption)	mutation <i>Acads^{del-J}</i>	Smith Richards et al., 2004
C57BL/6				
	C57BL/6J vs C57BL/6N	retinal dysplasia	mutation <i>rd8</i> of gene <i>Crb1</i>	Mattapallil et al., 2012
	C57BL/6J vs C57BL/6NCrl	insulin secretion, glucose tolerance, diet induced adipositas	deletion of exons 7 –11 of gene <i>Nnt</i>	Toye et al., 2005 Aston-Mourney et al., 2007 Wong et al., 2010
	C57BL/6J vs C57BL/6NCrl	alcohol consumption (higher alcohol preference of the J substrain as compared to the NCrl substrain)	unknown	Ramachandra et al., 2007 Mulligan et al., 2008
	C57BL/6JOla, C57BL/6NHsd vs C57BL/6NCrl	pilocarpine induced epilepsy	unknown	Müller et al., 2009

Inbred strain	Substrains	Phenotype	Genetic locus affected	Reference
C57BL/6				
	C57BL/6J vs C57BL/6N vs C57BL/6CrSlc	behaviour	unknown	Bothe et al., 2004 Bryant et al., 2008
	C57BL/6JNmj vs C57BL/6JOLA-Kun	neuro-anatomical structures	unknown	Jamot et al., 1994
C3H				
	C3H/HeJ	lipopolysaccharide resistant	mutation <i>Tlr4</i> ^{Lps-d}	Dumont, 1978
CBA				
	CBA/H	differences to other CBA strains affecting the hemopoetic system, behaviour, immune system, mortality, growth, and cell morphology	mutation <i>fm</i>	Hulse, 1965 http://jaxmice.jax.org/strain/000656.html
	CBA/Ki CBA/J	retinal degeneration	mutation <i>Pde6</i> ^{rd1}	Keeler, 1924 http://jaxmice.jax.org/strain/000656.html
	CBA/J vs CBA/Ca		variation of genes <i>Pgm-1</i> and <i>rd</i> .	Roderick, 1978
	CBA/J vs CBA/CaJ	not histocompatible	MiHC	Green und Kaufer, 1965
	CBA/N vs CBA/CAnN	differential expression of different surface antigens, different antibody responses		http://jaxmice.jax.org/strain/000656.html
	CBA/Ki vs CBAS ^t Ki	rejection of skin transplants rejection of tumors different incidences for spontaneous tumors, retinal degeneration in the CBA/Ki substrain, variation in food intake, induced obesity	MiHC	http://jaxmice.jax.org/strain/000656.html
DBA				
	DBA/1 vs DBA/2		genetic variants of <i>Gpd-1</i>	Roderick, 1978
	DBA/2J	resistance / susceptibility to various diseases	<i>Ahr</i> ^d	http://jaxmice.jax.org/strain/000671.html
	DBA/2J	impaired hearing	<i>Fscn2</i> ^{AhiB}	Johnson et al., 2008 Shin et al., 2010
	DBA/2J	impaired seeing	mutations <i>Gpnb</i> ^{R150X} , <i>Tyrp1</i> ^{isa} , <i>Myo5a</i> ^d	Hearing et al., 1973 Chang et al., 1999
	DBA/2J	Cd94 deficient	deletion in <i>Klr1d1</i>	Wilhelm et al., 2003

6 Different phenotypes of substrains

The specific phenotype of isogenic animal strains is in many cases not only determined by the differential alleles (as knock-out alleles or transgenes) but is also influenced by the genetic background.

An initial and helpful overview with regard to specific genotype and phenotype characteristics of common strains and substrains is supplied in the official information material of commercial companies such as *The Jackson Laboratory*. Further information can be extracted from the current databanks (see also Table 2).

The published attributes of animal strains should be tested prior to a series of experiments being planned or initiated in order to ensure the suitability of the strain to answer specific scientific questions. For example it is known that C57BL/6J mice have a high preference for alcohol and morphine (Melo et al. 1996; Philipps et al. 1994) which must be considered when an addictive behaviour experiment is being planned using these substances. In contrast C57BL/6NCrl animals consume smaller amounts of alcohol when available *ad libitum* but exhibit a robust alcohol deprivation effect, i.e. after a time period of de-habituation they start to consume considerably more alcohol which is indicative of a high-level addictive behavior with increased risk of relapses (Khisti et al. 2006).

Phenotypic specialties of strains and their respective substrains can affect all organs and functions and thus cannot be listed completely within the frame of this publication. To provide a first insight as to how broad the spectrum of variation is we would like to mention high preference for alopecia (Sundberg et al. 1994), microphthalmia (Smith et al. 1994), diet-induced obesity (Rossmeisl et al. 2003) or hydrocephalus (Festings: <http://www.informatics.jax.org/external/festing/mouse/docs/C57BL.shtml>) as well as variable fear responses (Radulovic et al. 1998; Stiedl et al. 1999), high incidence of mammary carcinoma (Hoag, 1963), extreme intolerance against alcohol and morphine (Phillips et al. 1994), increased sensitivity for audiogenically induced seizures (Fuller and Sjursen, 1967) and substrain specific incidence of hydronephrosis (Iglauer et al. 1996). An impressive example for the fatal effects of a substrains specific phenotype was described by Mattapallil et al. in 2012. Scientists who tried to investigate the role of specific genes in ocular disease by using various mouse models observed an unexpected inheritance pattern of the murine phenotypes. If animals were backcrossed to the C57BL/6 background an ocular phenotype was also frequently observed in control animals. As a consequence the possibility was considered that the “putative knock-out phenotypes” were not induced by the respective candidate genes but by alternative factors. Intensive literature search was performed which showed similarities to a retinal phenotype induced by the rd8 mutation of the *Crb1* gene already published in 2003 (Mehalow et al. 2003). The mutation is caused by deletion of a single nucleotide which results in a clinically relevant phenotype, characterized by morphologically altered areas in the ocular background which histologically correspond to foldings of the retina as well as retinal dysplasia and degeneration. Systematic PCR analyses were conducted and showed that the rd8 mutation had been fixed in the homozygous form in the genome of all C57BL/6N substrains used in this study. As a consequence all commercially available B6 strains and B6-derived ESC cells were screened. It was found that all C57BL/6N strains of all commercial suppliers carried the rd8 mutation; whereas all C57BL/6J substrains did not carry the defect. Taking into account the history of the C57BL/6 mouse strain the mutation can only have occurred after 1951 since the strain was separated into the J (The Jackson Laboratory) and the N (National Institute of Health) substrains at that stage.

In this context it must also be stated that the transgenic and knock-out mouse models of the *Knock-out Mouse–Projects of the University of California* (KOMP, <http://www.komp.org/>) and of the *European Conditional Mouse Mutagenesis Programs* (EUCOMM; <http://www.knockoutmouse.org/about/eucomm>) have been generated on the basis of C57BL/6N ES cells and thus without exception carry the rd8 mutation.

7 Substrains and standardization of animal experiments

Standardization of experiments is an important prerequisite for obtaining valid research results. With regard to animal experiments it is essential to consider environmental influences, the microbiological status and the genetic properties of the laboratory animals involved. In view of the broad multitude of laboratory rodent strains with specific mutations applied in research today, adequate genetic characterization of the models is mandatory.

Genetically modified strains are frequently exchanged between institutes without adequate information being provided with regard to the genetic background, such as strain or substrain affiliation. Likewise different mutations are frequently combined in one single strain without regard to the genetic background of the progenitor strains, hence leading to a non-defined or non-standardized genetic background of the resulting multi-mutant strain. The consequence is that it is not at all possible to find a suitable control strain for the multi-mutant variant.

Therefore, it is urgently advised before starting an animal experiment to thoroughly investigate the background history of the strains to be used. In the case that clear information cannot be provided the genetic background should be tested by the researcher responsible for the planned experiment.

In this context the enormous importance of correct strain and substrain nomenclature as well as accurate breeding documentation must be pointed out.

8 Literature

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