Variation of nitrate reductase genes in selected grass species

Jizhong Zhou, Andrzej Kilian, Robert L. Warner, and Andris Kleinhofs

Abstract: In order to study the variation of nitrate reductase (NR) genes among grass species, gene number, intron size and number, and the heme-hinge fragment sequence of 25 grass species were compared. Genomic DNA cut with six restriction enzymes and hybridized with the barley NAD(P)H and NADH NR gene probes revealed a single NAD(P)H NR gene copy and two or more NADH NR gene copies per haploid genome in most of the species examined. Major exceptions were *Hordeum vulgare*, *H. vulgare* ssp. *spontaneum*, and *Avena strigosa*, which appeared to have a single NADH NR gene copy. The NADH NR gene intron number and lengths were examined by polymerase chain reaction amplification. Introns I and III appeared to be absent in at least one of the NADH NR genes in the grass species, while intron II varied from 0.8 to 2.4 kilobases in length. The NADH NR gene heme-hinge regions were amplified and sequenced. The estimated average overall nucleotide substitution rate in the sequenced region was 7.8×10^{-10} substitutions/site per year. The synonymous substitution rate was 2.11×10^{-9} substitutions/synonymous site per year and the nonsynonymous substitution rate was 4.10×10^{-10} substitutions/nonsynonymous site per year. Phylogenetic analyses showed that all of the wild *Hordeum* species examined clustered in a group separate from *H. vulgare* and *H. vulgare* ssp. *spontaneum*.

Key words: nitrate reductase gene, gene copy number, intron, molecular phylogeny, grasses.

Résumé : Afin d'étudier la variation des gènes de la nitrate réductase (NR) chez les graminées, le nombre de gènes, le nombre et la taille des introns ainsi que la séquence de la région charnière ont été comparés chez 25 espèces de graminées. Lorsque digérés à l'aide de six enzymes de restriction et hybridés avec les gènes NAD(P)H-NR et NADH-NR de l'orge, les ADNs génomiques présentaient une seule copie du gène NAD(P)H-NR et deux copies ou plus du gène NADH-NR par génome haploïde chez la plupart des espèces examinées. Les principales exceptions à cette règle étaient *Hordeum vulgare*, *H. vulgare* ssp. *spontaneum* et *Avena strigosa*, qui semblent ne posséder qu'une seule copie du gène NADH-NR. Pour le gène NADH-HR, le nombre d'introns et leur taille ont été analysés par réaction de polymérisation en chaîne. Les introns I et III semblent être absents chez au moins un des gènes NADH-NR chez les graminées, tandis que l'intron II mesure entre 0,8 et 2,4 kilobases. Les régions charnières du gène NADH-NR ont été amplifiées et séquencées. Le taux moyen de substitutions a été estimé à $7,8 \times 10^{-10}$ substitutions par site et par année dans la région séquencée. Le taux de substitutions synonymes était de $2,11 \times 10^{-9}$ substitutions par site synonyme et par année et le taux de substitutions non-synonymes était de $4,10 \times 10^{-10}$ substitutions par site non-synonyme et par année. L'analyse phylogénétique a démontré que toutes les espèces sauvages examinées chez le genre *Hordeum* appartiennent à un même groupe distinct de celui formé par le *H. vulgare* et le *H. vulgare* ssp. *spontaneum*.

Mots clés : gène de la nitrate, réductase, nombre de copies du gène, introns, phylogénie moléculaire, graminées.

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Introduction

Nitrate assimilation is a fundamental process of higher plants and algae, as well as many fungi and bacteria. The reduction of nitrate to nitrite is catalyzed by nitrate reductase (NR) as the initial step in nitrate assimilation and is considered rate limiting (Beevers and Hageman 1969; Hewitt 1975). In eukaryotes, three types of reduced pyridine nucleotide dependent NRs are known: NADH specific (EC 1.6.6.1), NAD(P)H bispecific (EC 1.6.6.2), and NADPH specific (EC 1.6.6.3) (Guerrero et al. 1981). The NADH-specific NR is common in higher plants, while NAD(P)H-bispecific NR is found in soybean (Streit et al. 1985), maize (Redinbaugh and Campbell 1981), rice (Shen et al. 1976), and barley (Dailey et al. 1982). Single NADH and NAD(P)H NR genes are known in *Hordeum vulgare*, while *Oryza* and *Triticum* appear to have at least two NADH NR genes per haploid genome (Hamat et al. 1989; Kleinhofs et al. 1988).

NR is a homodimer with each subunit composed of three functional domains binding FAD, heme, and molybdenum cofactor in a 1:1:1 ratio (Redinbaugh and Campbell 1985). The FAD and heme functional domains are separated by short proteolytically sensitive hinge regions (Kubo et al. 1988). The amino acid sequence of the heme domain is highly conserved among species, while that of the FAD – heme-hinge region shows lower identity (Kleinhofs and Warner 1990; Campbell and Kinghorn 1990).

Higher plant NR genes have variable numbers and sizes of introns but the intron positions are conserved. NADH NR genes in tobacco and rice have three introns (Vaucheret et al. 1989; Choi et al. 1989), while barley has one large intron in the second position (Schnorr et al. 1991). The barley NAD(P)H NR gene has two small introns in the first and second intron positions (Miyazaki et al. 1991).

Although NR genetics, biochemistry, and molecular biology have been studied intensively in *H. vulgare*, they are little known in other grass species (for a detailed review see Kleinhofs and Warner 1990). In order to study the variation of NR genes in grasses, we examined the gene number, intron number and size, and the heme-hinge region sequences in 25 grass species using Southern blot and polymerase chain reaction (PCR) techniques and DNA sequencing.

Materials and methods

Plant species

For this study 25 diverse grass species were selected.

Species	Abbreviation	Ploidy	Source[a]
H. chilense Roemer & Schultes	Hch	2*x*	PBI
H. bogdanii Wilensky	Hbo	2*x*	PBI
H. stenostachys Godron	Hst	2*x*	PBI
H. murinum ssp. *glaucum* (Steudel) Tzvelev	Hgl	2*x*	PBI
H. marinum Hudson ssp. *marinum*	Hma	2*x*	PBI
H. pusillum Nuttal	Hpu	2*x*	PBI
H. murinum ssp. *leporinum* (Link) Arcangeli var. *simulans* Bowden	Hsi	6*x*	PBI
H. lechleri (Steudel) Schenck	Hle	2*x*	PBI
H. vulgare ssp. *spontaneum* (C. Koch) Thell., PBI 3733	Hsp	2*x*	PBI
H. vulgare ssp. *vulgare* L. cv. Steptoe, Az32	Hvu	2*x*	AK
H. bulbosum L.	Hbu	2*x*	PH
Triticum monococcum L.	Tmo	2*x*	CK
Sorghum bicolor L.	Sbi	2*x*	EP
Secale cereale L.	Sce	2*x*	EP
Pennisetum americanum (L.) Leeke	Pam	2*x*	CK
Avena strigosa Schreb.	Ast	2*x*	CK
Oryza sativa L., M201	Osa	2*x*	TO
Bromus arvensis L. (Iran)	Bar	2*x* (?)	RJ
Agropyron cristatum (L.) Gaertn. (Canada)	Acr	2*x* (?)	RJ
Festuca ovina L. (China)	Fov	2*x* (?)	RJ
Phalaris arundinacea L. (U.S.S.R.)	Par	2*x* (?)	RJ
Psathyrostachys juncea (Fischer) Nevcski (U.S.A.)	Pju	2*x* (?)	RJ
Lolium perenne L.	Lpe	2*x* (?)	WJ
Poa trivialis L.	Ptr	2*x* (?)	WJ
Bambusa vulgaris Schrad.	Bvu	?	JS

[a]PBI, Plant Breeding Institute, Cambridge, England; AK, Andris Kleinhofs, Washington State University (WSU); CK, Calvin Konzak, WSU; EP, Enrique Polle, WSU; TO, Thomas Okita, WSU; RJ, Richard Johnson, WSU; WJ, William Johnston, WSU; PH, Pat Hayes, Oregon State University; JS, John Stiles, University of Hawaii at Manoa.

All species were assumed to be diploid except *H. murinum* ssp. *leporinum* var. *simulans*, which is hexaploid. Data confirming the chromosome numbers for the forage, turf, and bamboo species, however, were not available.

DNA isolation

Total DNA was extracted from 0.25 to 0.35 g of freeze-dried leaf tissue from plants 12–45 days old generated from a single seed, except for the bamboo species where total DNA was extracted from dried leaves provided by Dr. J. Stiles, University of Hawaii at Manoa. Leaves were ground to a fine powder in liquid nitrogen with sand using a mortar and pestle, suspended in 20 mL of prewarmed (65°C) buffer containing 100 mM Tris–HCl (pH 8.0), 50 mM EDTA, 1.4 M NaCl, and 1% (w/v) CTAB (cetyltrimethylammonium bromide). Proteinase K was added to a final concentration of 50 μg/mL and the sample was mixed vigorously. Then, 2.0 mL of 20% (w/v) SDS was added and the sample was incubated at 65°C for 2 h with gentle inversion every 15 min. The sample was extracted twice with 20 mL equal parts of phenol and chloroform – isoamyl alcohol (24:1), centrifuged, and the DNA precipitated from the aqueous phase with 0.6 volumes

Table 1. Oligonucleotide primers used for amplification by PCR.

Targets	Primer sequences	Designation[a]
Heme-hinge	5′GCTCAAGCGGAGCACGTCCA3′	NR 1560 + 20
	5′CCTTGGTGGCCTCGCGGAT3′	NR 1919 − 19
Intron I	5′GAGCATGAGCCGGGAGTGGG3′	NR 771 + 20
	5′TTGATGGTGTATGCGCGCTGTG3′	NR 1145 − 22
Intron II	5′CACAGCGCGCATACACCATCAA3′	NR 1124 + 24
	5′CCTCCACTCGTGTGATCTTCTTGCC3′	NR 1192 − 25
Intron III	5′TGTGCACGCTCGACATCCCG3′	NR 1223 + 20
	5′CTTGTCCCCGGCGGTGTTCA3′	NR 1608 − 20

[a]The primer designation numbers correspond to the barley NADH nitrate reductase gene cDNA sequence (accession Number X57844).

of isopropanol. The precipitated DNA was spooled out, washed with 70% ethanol, dissolved in 4 mL of sterile distilled water containing 80 μg of heat treated RNase A, and incubated for 2 h at 37°C. The sample was extracted with 4 mL of chloroform. The DNA was precipitated with 0.1 volumes of 3 M sodium acetate and 2 volumes of 95% ethanol, washed with 70% ethanol, and dissolved in an appropriate volume of 1× TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), and stored at 4 °C.

Polymerase chain reaction

NADH NR specific oligonucleotide primers were designed using the barley NADH NR gene sequence (Schnorr et al. 1991) and the OLIGO program (Rychlick and Rhoads 1989) (Table 1). The oligonucleotides for amplifying the NADH NR gene heme-hinge region were from National Biosciences (Hamel, Minn.). The oligonucleotides for amplifying NADH NR gene introns were synthesized by the Laboratory of Biotechnology and Bioanalysis at Washington State University.

All stocks for PCR amplification were made and procedures performed with the precautions suggested by Kwok and Higuchi (1989). A 0.5 μg genomic DNA sample was added to a standard PCR mixture containing 1× Taq polymerase buffer, 1.25 U Taq polymerase (Promega, Madison, Wis.), 100 μmoles dNTPs, and 20 pmoles of each primer in a 50 μL reaction. The reaction conditions for amplification of the NADH NR gene heme-hinge region (359 base pairs (bp)) were as follows: denaturation at 94°C for 4 min, 35 cycles of 94°C for 1 min, 62.5°C for 1 min, and 72°C for 1 min. Samples were then subjected to a 10 min incubation at 72°C before being stored at 7°C. The reaction conditions for the amplification of the NADH NR gene intron regions were as described above except that different annealing temperatures (56, 58, and 60°C) were used and the extension time at 72°C was increased to 2 min. Temperature cycling was performed in a programmable temperature cycler (Model 50, COY Inc., Ann Arbor, Mich.).

Southern blotting

A 10 μg genomic DNA sample was digested with restriction enzymes, *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, and *Xba*I, separated in an 0.8% agarose gel, and transferred to GeneScreen Plus membrane (DuPont, Boston, Mass.). Prehybridization, hybridization, and washings were carried out according to Kleinhofs et al. (1993) except that 0.1× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate) and 1% SDS was used for the final washing at 65°C. A 359-bp heme-hinge fragment amplified from the barley NADH NR gene was cloned, designated pJZ1, and used as a probe to detect barley NADH and NAD(P)H NR genes at high stringency. The specific NAD(P)H NR gene probe was the 440-bp *Sph*I–*Sac*II fragment from the 5′ region of the barley NAD(P)H NR gene clone pMJ4.1 (Miyazaki et al. 1991).

Heteroduplex mobility shift analyses

The amplified 359-bp heme-hinge fragment was examined by heteroduplex mobility shift analysis (White et al. 1992). The PCR reaction mixture and reaction conditions were as described above except that the mixture contained 0.1 μL [^{32}P]dCTP (3000 Ci/mmol, 10 μCi/μL (1 Ci = 37 GBq)). A 7-μL aliquot of the PCR product was heated for 5 min at 95°C, slowly cooled to room temperature, and analyzed by gel electrophoresis (5% polyacrylamide (19 parts acrylamide : 1 part bisacrylamide) and 1% glycerol) for 12 h at 3 W constant power.

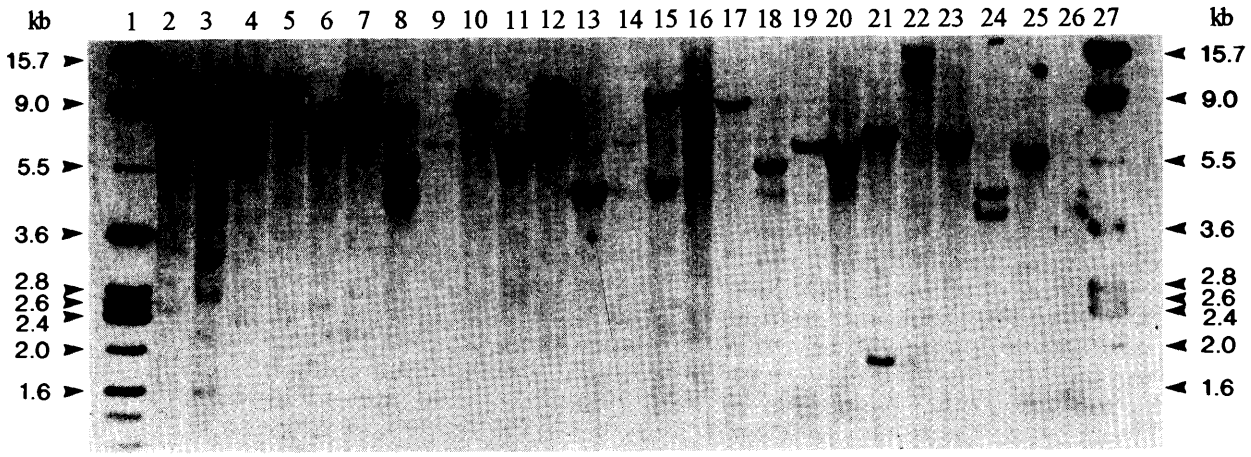
DNA sequencing

Two direct DNA sequencing methods were used to determine the DNA sequence of the NADH NR gene heme-hinge fragment. Single-stranded DNA was generated and sequenced using T7 polymerase (Gyllensten and Erlich 1988). The sequences from the opposite direction were determined using Taq polymerase (Adams and Blakesley 1991).

Direct DNA sequencing with T7 polymerase

The DNA sequence of the antisense strand of the NADH NR gene heme-hinge fragment was determined by the dideoxy chain termination method (Sanger et al. 1977) using the T7 sequencing kit (Pharmacia, Piscataway, N.J.). A 5-μL (20–30 ng) aliquot of the amplified DNA was added to the polymerase chain reaction mixture containing 1× Taq buffer, 200 nM dTNPs, 5 U Taq polymerase (Promega, Madison, Wis.), 25 pmoles of the primer (NR 1560+20) in a 100 μL reaction. The reaction conditions for single-stranded DNA amplification were the same as those for double-stranded DNA amplification except that 40 cycles were used. The single-stranded PCR products were purified according to Brow (1990). One-fourth to one-half of the

Fig. 1. Southern blot showing hybridization of grass species genomic DNA with the barley NAD(P)H NR gene-specific probe. DNA was digested with *Dra*I. The final wash was at 65°C with 0.1× SSC plus 1% SDS. Lanes: 1 and 27, λDNA marker cut with *Hin*dIII, *Eco*RI, and *Bam*HI; 2, *H. chilense*; 3, *H. bogdanii*; 4, *H. stenostachys*; 5, *H. murinum* ssp. *glaucum*; 6, *H. marinum* ssp. *marinum*; 7, *H. pusillum*; 8, *H. murinum* ssp. *leporinum* var. *simulans*; 9, *H. lechleri*; 10, *H. vulgare* ssp. *spontaneum*; 11, *H. vulgare* ssp. *vulgare*; 12, *H. bulbosum*; 13, *T. monococcum*; 14, *S. bicolor*; 15, *S. cereale*; 16, *P. americanum*; 17, *A. strigosa*; 18, *O. sativa*; 19, *Bromus arvensis*; 20, *Agropyron cristatum*; 21, *Festuca ovina*; 22, *Phalaris arundinacea*; 23, *Psathyrostachys juncea*; 24, *Lolium perenne*; 25, *Poa trivialis*; 26, *Bambusa vulgaris*.



recovered DNA was used for sequencing. DNA sequencing was carried out according to the manufacturer's instructions except that, before the sequencing reaction was terminated, terminal deoxynucleotidyl transferase (TdT) was used to remove artifact bands (Fawcett and Barlett 1991). To determine the DNA sequence nearest the primers, manganese was included in Sequenase kit buffer according to the manufacturer's instructions (United States Biochemical Corporation, Cleveland, Ohio). Gel electrophoresis was carried out at 60 W constant power on a wedge gel made with 6% polyacrylamide and 42% urea or at 50 W constant power on a 7% Long Ranger gel (AT Biochem, Malvern, Pa.).

Direct DNA sequencing with Taq polymerase

The DNA sequence of the sense strand was determined by the dideoxy chain termination method using the double-stranded DNA cycle sequencing system (BRL, Gaithersburg, Md.). Double-stranded PCR products were purified by electrophoresis on a 1.5% agarose gel. The target band was excised and the DNA was recovered using PREP-A-Gene DNA purification kit (Bio-Rad, Richmond, Calif.) and used for sequencing according to the manufacturer's instructions. The DNA fragment was sequenced in a COY temperature cycler for 20 cycles at 95°C for 30 s, 61°C for 1 min, and 70°C for 1 min, and 10 cycles at 95°C for 30 s and 70°C for 1 min. Electrophoresis was performed at 60 W constant power on 6% polyacrylamide and 42% urea wedge gels.

Nucleotide sequence accession numbers

The NR gene nucleotide sequences determined for the grass species examined in this study were submitted to GenBank and assigned the following accession numbers: *Avena strigosa*, L40147; *Hordeum bogdanii*, L40148; *Hordeum chilense*, L40149; *Hordeum lechleri*, L40150; *Hordeum pusillum*, L40151; *Hordeum vulgare* ssp. *spontaneum*, L40152; *Hordeum stenostachys*, L40153; *Secale cereale*, L40154; and *Triticum monococcum*, L40155.

Data analyses

Sequence data were analyzed in a Digital system VAX 11/785 and 386-25 MHz personal computer. The number of nucleotide substitutions was computed by Nei and Gojobori's (1986) method using the computer program kindly provided by Dr. A. Hughes (Pennsylvania State University, University Park, Pa.) after the sequences had been aligned by the program PILEUP in Genetics Computer Group software package (Devereaux et al. 1984). The phylogenetic trees were constructed by the maximum parsimony method with 500 replicates of bootstrap analyses, the maximum likelihood method, and the UPGMA (unweighted pair-group method using arithmetic means) method using the programs in the PHYLIP phylogeny inference package (Felsenstein 1989).

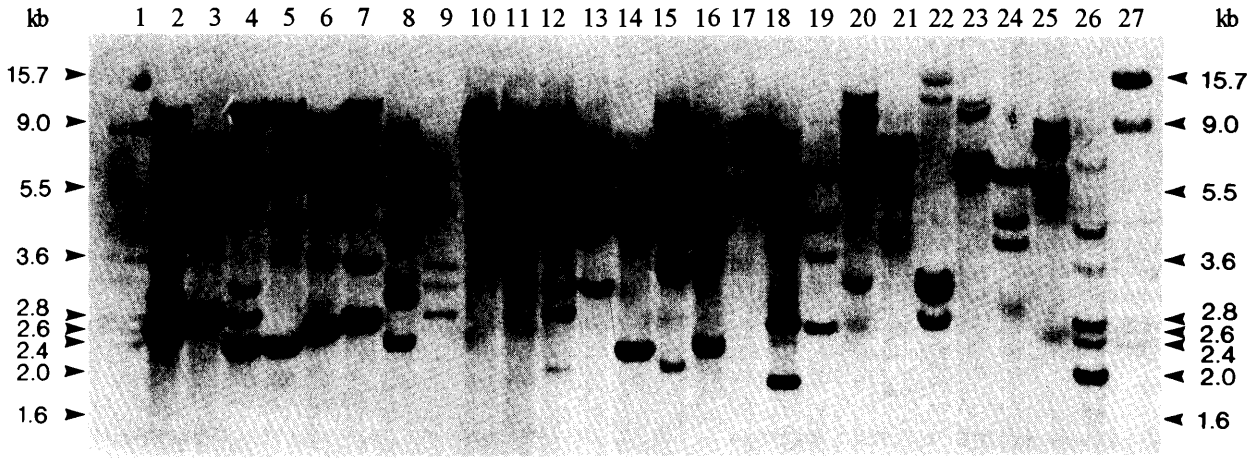
Results

NR gene copy number

DNA from various grass species digested with 6 restriction enzymes and hybridized with the NAD(P)H NR gene-specific probe showed one band in most species, suggesting the presence of one copy of the NAD(P)H NR gene per haploid genome (Fig. 1). The species showing more than one band may be polyploid (*H. murinum* ssp. *leporinum* var. *simulans*), heterozygous (*H. bulbosum*, *S. cereale*) or both. *Sorghum bicolor* and *Pennisetum americanum* failed to hybridize, indicating that these species may either not have or have a highly diverged form of the NAD(P)H NR gene.

The same blots hybridized with the barley NADH NR gene heme-hinge fragment probe showed 3–7 bands with

Fig. 2. Southern blot showing hybridization of grass species genomic DNA with the barley NADH NR gene-specific probe. DNA was digested with *Dra*I. The final wash was at 65°C with 0.1× SSC plus 1% SDS. Lanes: 1 and 27, λDNA marker cut with *Hin*dIII, *Eco*RI, and *Bam*HI; 2, *H. chilense*; 3, *H. bogdanii*; 4, *H. stenostachys*; 5, *H. murinum* ssp. *glaucum*; 6, *H. marinum* ssp. *marinum*; 7, *H. pusillum*; 8, *H. murinum* ssp. *leporinum* var. *simulans*; 9, *H. lechleri*; 10, *H. vulgare* ssp. *spontaneum*; 11, *H. vulgare* ssp. *vulgare*; 12, *H. bulbosum*; 13, *T. monococcum*; 14, *S. bicolor*; 15, *S. cereale*; 16, *P. americanum*; 17, *A. strigosa*; 18, *O. sativa*; 19, *Bromus arvensis*; 20, *Agropyron cristatum*; 21, *Festuca ovina*; 22, *Phalaris arundinacea*; 23, *Psathyrostachys juncea*; 24, *Lolium perenne*; 25, *Poa trivialis*; 26, *Bambusa vulgaris*.



2–6 bands being due to the NADH NR genes (Fig. 2). Major exceptions were *H. vulgare* and *A. strigosa*, which had only one NADH NR specific band. *Hordeum vulgare* ssp. spontaneum showed 4 bands when digested with *Bam*HI but only one band when digested with the other 5 enzymes. This apparent discrepancy may be due to partial digestion. These data suggest that there are two or more NADH NR genes per haploid genome in many grass species but only one NADH NR gene per haploid genome in *H. vulgare*, *H. vulgare* ssp. *spontaneum*, and *A. strigosa*.

NADH NR gene intron analyses

Intron number in grass species was determined by PCR amplification of the regions known to contain introns in other species. Primers spanning the barley NADH NR gene intron II yielded 1 or 2 fragments, when amplification was carried out at fairly stringent annealing conditions (58 or 60°C) (Table 2). All species examined appeared to have an intron in position II that varied in length from 0.8 to 2.4 kilobases (kb). In some species, the annealing stringency had to be reduced to 58°C in order to amplify a fragment. Reducing the annealing temperature resulted in amplification of a second unexpected DNA product of 0.8 kb in *H. vulgare* and *H. vulgare* ssp. *spontaneum*. This fragment is of unknown origin and may be an artifact or may be due to a previously undiscovered highly diverged NR gene sequence.

The regions where NADH NR gene introns I and III occur in some species were amplified at the annealing temperatures of 56, 58, and 60°C. From one to three PCR products were obtained for all species at 56°C, while only a product corresponding to the size of the barley NADH NR gene lacking the first and the third intron was obtained at

Table 2. NADH nitrate reductase gene intron II products produced by PCR amplification.

Species	Fragments (kb) 58°C	60°C
Hordeum chilense	1.5, 0.8	1.5
H. bogdanii	1.5, 0.8	1.5
H. stenostachys	1.7, 0.8	1.7
H. murinum ssp. *glaucum*	1.6, 1.2	1.6
H. marinum	1.8, 0.8	1.8
H. pusillum	1.6, 0.8	1.6
H. murinum ssp. *leporinum* var. *simulans*	2.1, 1.6	2.1
H. lechleri	2.2, 0.8	2.2
H. vulgare ssp. *spontaneum*	1.7, 0.8	1.7
H. vulgare ssp. *vulgare*	1.7, 0.8	1.7
H. bulbosum	1.7, 0.8	1.7
Triticum monococcum	2.4, 1.5	2.4
Sorghum bicolor	1.6, 0.8	1.6
Secale cereale	2.1, 1.5	2.1
Pennisetum americanum	0.8	—
Avena strigosa	1.7	—
Oryza sativa	0.8	—
Bromus arvensis	1.9	1.9
Agropyron cristatum	1.7	—
Festuca ovina	2.4	—
Phalaris arundinacea	2.1	2.1
Psathyrostachys juncea	2.0	2.0
Lolium perenne	1.5	1.5
Poa trivialis	1.1	—
Bambusa vulgaris	1.7	—

Table 3. Nucleotide substitutions of NADH NR gene heme-hinge fragment among the grass species examined.

	Hch	Hbo	Hst	Hpu	Hle	Hsp	Hvu	Tmo	Sce	Ast	Osa
Hch		0.19	0.06	0.08	0.06	0.75	0.71	0.27	0.39	0.26	0.41
Hbo	0.02		0.19	0.10	0.15	0.40	0.37	0.23	0.35	0.20	0.23
Hst	0.01	0.01		0.08	0.06	0.75	0.71	0.32	0.38	0.31	0.39
Hpu	0.01	0.01	0.00		0.05	0.60	0.57	0.21	0.28	0.22	0.29
Hle	0.01	0.01	0.00	0.00		0.67	0.63	0.25	0.36	0.26	0.34
Hsp	0.06	0.05	0.06	0.06	0.06		0.02	0.48	0.63	0.49	0.34
Hvu	0.06	0.05	0.06	0.06	0.06	0.00		0.45	0.59	0.46	0.37
Tmo	0.02	0.02	0.02	0.02	0.02	0.02	0.02		0.40	0.16	0.23
Sce	0.05	0.03	0.03	0.03	0.03	0.05	0.05	0.04		0.44	0.40
Ast	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.04		0.19
Osa	0.06	0.04	0.05	0.05	0.05	0.06	0.06	0.04	0.05	0.03	

Note: Synonymous substitutions per synonymous site of NADH nitrate reductase gene heme-hinge region among the grass species examined are shown above the diagonal and nonsynonymous substitutions per nonsynonymous site are shown below the diagonal. See Material and methods for species abbreviations. The rice and barley NADH NR gene sequences are from Choi et al. (1989) and Schnorr et al. (1991), respectively.

58 and 60°C. Thus, the grass species investigated appear to have at least one NADH NR gene similar to the barley NADH NR gene in that introns I and III are absent.

Homogeneity of the PCR amplified NADH NR gene heme-hinge region

In order to estimate divergence of the NADH NR gene sequences among grass species, we amplified and sequenced a 359-bp DNA fragment from the NADH NR gene heme-hinge region. The DNA fragment amplified at high stringency (62.5°C annealing temperature) was the same length in all species examined. Since multiple gene copies are present in some species (Fig. 2), the homogeneity of the amplified PCR products was examined by heteroduplex mobility shift analysis (White et al. 1992). In most cases the PCR amplified 359-bp fragment was heterogeneous (data not shown). These results are in agreement with the Southern blot analyses, which indicate the presence of multiple NADH NR genes in many grass species. The PCR amplified products of *H. chilense* and *T. monococcum* appeared to be homogeneous, which is not consistent with the Southern blot analyses. This may be because only one NADH NR gene was amplified, because the two NADH NR genes are nearly identical in this region, or because the sensitivity of the method was not high enough to distinguish different products. In most cases, the different NADH NR genes were not amplified equally, presumably because of the sequence divergence of the primer binding regions in different NADH NR genes. The PCR amplified fragments showing 80% or higher homogeneity were sequenced.

Nucleotide substitution

The number of nucleotide substitutions among the grass species was determined with 55 pairwise sequence comparisons (Table 3). The number of synonymous substitutions per synonymous site varied greatly among the grass species (0.02–0.75), with an average of 0.341 ± 0.195, presumably because of the differences in divergence time among species. Among all comparisons, the highest synonymous substitution values were obtained for the comparisons of *H. vulgare* and *H. vulgare* ssp. *spontaneum* with the other *Hordeum* species. The number of nonsynonymous substitutions per nonsynonymous site among species ranged from 0 to 0.07 with an average of 0.033 ± 0.02. Similar to synonymous substitutions, the number of nonsynonymous substitutions was the highest between *H. vulgare* and *H. vulgare* ssp. *spontaneum* and the other *Hordeum* species, and the lowest among the other wild *Hordeum* species. On average, the number of synonymous substitutions was about 10 times greater than nonsynonymous substitutions. There was a significant positive correlation between synonymous and nonsynonymous substitutions ($r = 0.51$, $P < 0.001$).

To consider absolute rates of nucleotide substitutions, the divergence times between species are needed. Unfortunately, owing to the paucity of the plant fossil record, only rough estimates of divergence times are available. The divergence time between Pooideae, Panicoideae, and Oryzoideae has been estimated as 50–70 Ma (Stebbins 1981; Wolfe et al. 1989). *Triticum*, *Hordeum*, *Secale*, and *Avena* are classified in the subfamily of Pooideae, whereas *Zea* is in Panicoideae, and *Oryza* is in Oryzoideae (Gould 1968). The substitution rates between Pooideae and Panicoideae or Oryzoideae lineages were estimated by $K/2T$ in which K is the number of nucleotide substitutions per site and T is the divergence time (Table 4). We used 70 Ma as the divergence time between Pooideae, Panicoideae, and Oryzoideae. The rates of synonymous, nonsynonymous, and overall nucleotide substitutions showed considerable variation (20–40%) among the Pooideae lineage, while they were very similar among Panicoideae and Oryzoideae lineages (Table 4). The average synonymous substitution rate of the NADH NR gene heme-hinge region was estimated to be 2.11×10^{-9} substitutions/synonymous site per year, while the average nonsynonymous substitution rate was 4.10×10^{-10}

Table 4. Rates of nucleotide substitution per site per year ($\times 10^{-9}$).

Species	*Oryza sativa*			*Zea mays*		
	SNS	NNS	ONS	SNS	NNS	ONS
Hordeum chilense	1.86	0.44	0.93	0.91	0.62	1.02
H. bogdanii	1.63	0.31	0.59	1.57	0.54	0.76
H. stenostachys	2.76	0.34	0.81	2.66	0.57	0.99
H. pusillum	2.08	0.34	0.69	1.84	0.57	0.84
H. lechleri	2.41	0.34	0.75	2.16	0.57	0.90
H. vulgare ssp. *spontaneum*	2.46	0.43	0.84	2.99	0.43	0.93
H. vulgare ssp. *vulgare*	2.63	0.43	0.87	2.81	0.43	0.90
Triticum monococcum	1.61	0.25	0.54	1.38	0.26	0.51
Secale cereale	2.86	0.38	0.86	3.36	0.51	1.04
Avena strigosa	1.37	0.18	0.44	0.91	0.23	0.39
Mean	2.17	0.35	0.73	2.06	0.48	0.83
SD	0.53	0.08	0.16	0.87	0.14	0.22

Note: SNS, synonymous nucleotide substitution; NNS, nonsynonymous nucleotide substitution; ONS, overall nucleotide substitution; SD, standard deviation. The maize NADH NR gene sequence is from Gori and Campbell (1989).

substitutions/nonsynonymous site per year. The average overall nucleotide substitution rate was 7.8×10^{-10} substitutions/site per year.

Molecular phylogeny

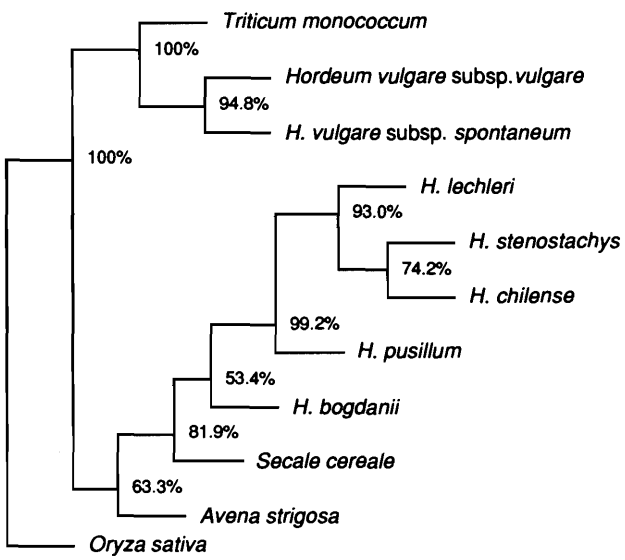
The maximum parsimony tree showed that all the wild *Hordeum* species examined clustered in a group separate from *H. vulgare* and *H. vulgare* ssp. *spontaneum*, and appeared to be more closely related to *A. strigosa* than to *H. vulgare* and *H. vulgare* ssp. *spontaneum* (Fig. 3). An identical tree topology was obtained by the maximum likelihood method and a similar tree was obtained by UPGMA (not shown).

Discussion

Southern blot analyses indicated that a single copy of the NAD(P)H NR gene and two or more copies of NADH NR genes per haploid genome occur in most of the grass species investigated. These observations are in agreement with previous data showing two NADH NR genes per haploid genome in *T. aestivum* (Kleinhofs et al. 1988) and *Oryza sativa* (Hamat et al. 1989), but are surprising in that only a few species have a single NADH NR gene.

Southern blot and heteroduplex mobility shift analysis showed that rice has at least two, and possibly three, NADH NR genes. Hamat et al. (1989) reported that the *japonica* rice cultivar M201 had at least three, and possibly as many as six, NR genes with homology to the barley NADH NR gene. Two of these genes have been cloned and sequenced (Choi et al. 1989). High sequence identity was observed between one of the rice NADH NR genes and the barley NADH NR gene. This rice gene has three introns of 108, 1954, and 85 bp (Choi et al. 1989). The other gene had high identity to a small 3′ region of the barley NADH NR gene but no other homology could be detected, suggesting that it may not be a functional gene (Choi et al. 1989). Amplification of the rice NR introns in this study yielded

Fig. 3. Evolutionary relationships of grass species based on the NADH NR gene heme-hinge sequences. The parsimony tree was constructed using DNABOOT with rice as the outgroup. The percentages indicate confidence levels based on 500 bootstrap replicates.



fragments suggesting the absence of introns I and III and a 0.8-kb intron II. These data suggest that an NADH NR gene that has not yet been cloned may exist in rice.

The average overall substitution rate of the analyzed NADH NR gene heme-hinge region (7.8×10^{-10}) is higher than that estimated for alcohol dehydrogenase 1 (5.18×10^{-10}) (Gaut and Clegg 1991) and for *RbcL* ($5.0–6.5 \times 10^{-10}$) (Doebley et al. 1990). The synonymous substitution rate of this region (2.11×10^{-9}) is lower than that of alcohol dehydrogenase 1 (7.9×10^{-9}) and is below the range of synonymous substitutions estimated for plant nuclear genes ($5–30 \times 10^{-9}$) (Wolfe et al. 1987). The nonsynonymous

substitution rate of this region (4.1×10^{-10}) is above that of alcohol dehydrogenase 1 (2.5×10^{-10}). The higher overall and nonsynonymous substitution rate may be due to rapid divergence of the hinge region. In addition, there are 2 or more copies of NADH NR genes in most of the grass species examined. Heteroduplex mobility shift analyses showed that the majority of the PCR products were amplified from one NADH NR gene, but we were not able to determine which of the NADH NR genes was amplified. Presumably, the gene copy that was more closely related to the barley NADH NR gene was amplified and sequenced.

The phylogeny of the NADH NR gene heme-hinge sequences, analyzed by maximum parsimony method, maximum likelihood method, and UPGMA showed that *H. vulgare* and *H. vulgare* ssp. *spontaneum* occur in a group separate from the other *Hordeum* species. The separation of the *Hordeum* species into two clusters is in agreement with the isozyme (Jorgensen 1986), chloroplast DNA (Doebley et al. 1992), and repetitive DNA (Svitashev et al. 1994) studies on *Hordeum* species and with the genomic classification proposal of Dewey (1984) placing *H. vulgare*, *H. vulgare* ssp. *spontaneum*, and *H. bulbosum* in a separate genus from the other *Hordeum* species.

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