Biochemical Genetics, Vol. 24, Nos. 5/6, 1986

Pig Mitochondrial DNA: Polymorphism, Restriction Map Orientation, and Sequence Data

Tomomasa Watanabe,[1] Yukimasa Hayashi,[2] Jun Kimura,[3] Yukio Yasuda,[4] Naruya Saitou,[5] Takeshi Tomita,[6] and Nobuaki Ogasawara[1]

Received 25 Sept. 1985—Final 17 Jan. 1986

Restriction endonuclease cleavage patterns of mitochondrial DNA (mtDNA) in pigs were analyzed using 18 enzymes which recognize six nucleotides and 1 four-nucleotide-recognizing enzyme. Pigs including Taiwan native breeds and miniature strains maintained in Japan were examined in this study; four commercial breeds of pigs and Japanese wild boars have been investigated earlier [Watanabe, T., et al. (1985). Biochem. Genet. *23:105]. mtDNA polymorphisms were observed in the cleavage patterns of five restriction enzymes,* Bgl*II,* Eco*RV,* Sca*I,* Stu*I, and* Taq*I. The results support the previous hypothesis that pigs must be derived from two different maternal origins, European and Asian wild boars, and that a breed, Large White, arises from both European and Asian pigs. Two* Hind*III cleavage fragments were cloned into the* Hind*III site of M13mp10 and were partially sequenced by the dideoxynucleotide-chain termination method. Furthermore,* Dra*I and* Stu*I cleavage sites were newly determined on the restriction endonuclease map. On the basis of these results, the restriction endonuclease cleavage map of pig mtDNA was rewritten. Comparing sequence data of pig mtDNA at 237 positions with those of cow, human, mouse, and rat mtDNA, the sequence*

[1] Department of Biochemistry, Institute for Developmental Research, Aichi Prefecture Colony, Kamiyacho, Kasugai, Aichi 480-03, Japan.
[2] Department of Genetics, Institute for Developmental Research, Aichi Prefecture Colony, Kamiyacho, Kasugai, Aichi 480-03, Japan.
[3] Medical Research Institute, Nippon Well-Being Foundation, Ikki-machi, Aizuwakamatsu, Fukushima 965, Japan.
[4] Central Institute for Experimental Animals, Nogawa, Miyamaeku, Kawasaki 211, Japan.
[5] Center for Demographic and Population Genetics, University of Texas, Health Science Center, Houston, Texas 77225.
[6] Laboratory of Animal Genetics, Faculty of Agriculture, Nagoya University, Furocho, Chikusa Nagoya 464, Japan.

0006-2928/86/0600-0385$05.00/0

difference, silent and replacement changes, and transitions and transversions among mammalian species were estimated. The relationships among them are discussed.

KEY WORDS: pigs; mitochondrial DNA; restriction endonuclease; East Asia.

INTRODUCTION

Since animal mitochondrial DNA (mtDNA) is maternally inherited and the evolutionary rate of nucleotide substitution is very rapid (Brown *et al.*, 1979, 1982), mtDNA polymorphism based on restriction endonuclease analysis has allowed us to clarify genetic relationships of various species. We are investigating mtDNA polymorphism of domestic animals and fowls (Watanabe *et al.*, 1985a, b, c; Wakana *et al.*, 1986). In pigs, polymorphism of mtDNA has been reported previously (Watanabe *et al.*, 1985a) and suggests that two types of mtDNAs, of European and Asian wild boar origins, exist in pigs. This paper describes the further investigation of mtDNA polymorphism in four Taiwan native breeds and three miniature strains maintained in Japan, the restriction endonuclease cleavage map orientation, and the sequence data.

MATERIALS AND METHODS

Animals. Four breeds of Taiwan native pigs were introduced into Japan in 1982 and are maintained at the Medical Research Institute, Nippon Well-Being Foundation. These breeds are Taoyaun, Meinung, Short-Ear, and Lanyu. The former two breeds originated from native pigs of South China, and the latter two from pigs of Southeastern Asia.

Three strains of miniature pigs are maintained in Japan. The Ohmini strain was originally developed from the small pigs of Manchuria in the Republic of China and are maintained at the Nippon Research Laboratory for Domestic Animals. The Göttingen miniature strain was introduced into the Central Institute for Experimental Animals from Germany. The Göttingen strain was developed from a cross of Homel miniatures with a small type of Vietnamese pig (Haring *et al.*, 1965), and the unpigmented gene was introduced by crossing with the Landrace breed. The Pitman-Moore strain was maintained at the Nippon Institute for Biological Science, originally developed from feral hogs from the southern United States.

Chemicals. Eighteen restriction endonucleases were purchased from Nippon Gene Co. or Takara Shuzo Co. In addition, *Dra*I was obtained from Pharmacia. [α-^{32}P]dCTP, the M13mp10 cloning kit, and the sequencing kit were purchased from Amersham. Alkaline phosphatase was from Boerhinger-

Mannheim Co. Other reagents were commercial preparations of the highest purity available.

MtDNA Preparations, Restriction Endonuclease Digestion, and Gel Electrophoresis. mtDNA was prepared from kidneys of pigs. The procedures for mtDNA preparation, restriction endonuclease digestion, agarose gel electrophoresis, and polyacrylamide gel electrophoresis have been described previously (Watanabe *et al.*, 1985a, c).

DNA Cloning and Sequencing. Pig mtDNA was digested with *Hind*III and was ligated with an alkaline phosphatase-treated vector, M13mp10 (Groneborn and Messing, 1978; Messing *et al.*, 1981). The ligation product was used to transform *Escherichia coli* JM105, in order to prepare a single-strand template. The sequence of a *Hind*III-digested fragment was determined by dideoxynucleotide-chain termination method using a flanking universal primer and [α-^{32}P]dCTP (Sanger *et al.*, 1977, 1980).

RESULTS AND DISCUSSION

Eighteen restriction endonucleases which recognize six base pairs were used in this study. mtDNA polymorphisms were detected in the cleavage patterns of 4 restriction enzymes, *Bgl*II, *Eco*RV, *Sca*I, and *Stu*I, but no difference was observed in the patterns of the other 14 restriction enzymes, *Bam*HI, *Bbe*I, *Dra*I, *Eco*RI, *Hind*III, *Kpn*I, *Mlu*I, *Pst*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Xba*I, and *Xho*I, among pigs examined in this study and in the previous one (Watanabe *et al.*, 1985a). As shown in Table I, mtDNA from all four breeds of Taiwan

Table I. Types of Restriction Endonuclease Cleavage Patterns in Four Native Breeds of Taiwan Pigs and Three Miniature Strains Maintained in Japan

Breeds	No. examined	Restriction pattern			
		*Bgl*II	*Eco*RV	*Sca*I	*Stu*I
Taiwan native breeds					
Taoyaun	1	B	A	B	B
Meinung	2	B	A	B	B
Short-ear	1	B	A	B	B
Lanyu	1	B	A	B	B
Miniature strains maintained in Japan					
Ohmini	2	B	A	B	B
Göttingen	2	B	A	B	B
Pitman-Moore	4	A	A	A	A

native pigs showed the cleavage types of B in *Bgl*II, A in *Eco*RV, B in *Sca*I, and B in *Stu*I. The cleavage types of mtDNA from two strains of miniature pigs, Ohmini and Göttingen, were also B–A–B–B in *Bgl*II–*Eco*RV–*Sca*I–*Stu*I, respectively. On the other hand, a miniature pig of the Pitman-Moore strain showed the cleavage type of mtDNA, A–A–A–A. *Dra*I was first used in this study, when seven fragments were observed in the cleavage patterns and the molecular weights were estimated as 6.54, 2.54, 2.29, 1.96, 1.52, 1.23, and 0.39 kb. Therefore, the percentage sequence divergence among pigs showing different cleavage patterns was recalculated with equations 10 and 16 of the report by Nei and Li (1979). An assumption of this method is that a cleavage-site difference is due to the substitution of one base pair. The percentage sequence divergence of mtDNA between A–A–A–A and B–A–B–B was 1.75, and that between A–A–A–A and A–B–A–A was 0.17. From these results, the relationships among various breeds of pigs and Japanese wild boars based on the restriction patterns are diagrammed in Fig. 1. The restriction patterns of Landrace, Hampshire, Duroc, Pitman-Moore and Large White I are classified as the European type. The patterns of Large White II, Ohmini, Göttingen, Taiwan native breeds, and Japanese wild boars are grouped as the Asian type. The time of divergence between both European and Asian types was calculated to be about 0.8–0.9 $\times$ 10^6 years. The hypothesis that the pigs examined in our studies are from different maternal origins, European and Asian wild boars, and that the breed, Large White, arises from both European and Asian pigs is strongly supported.

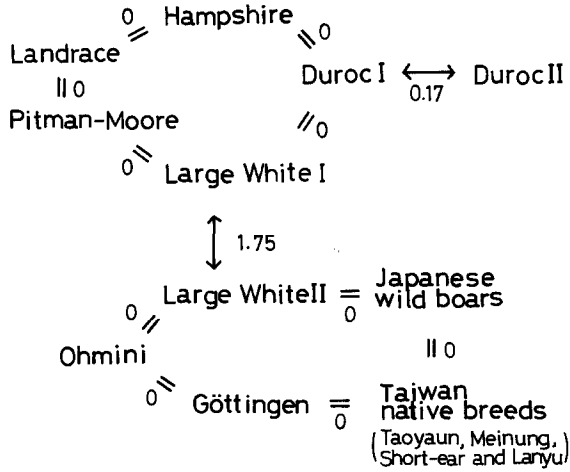

Fig. 1. The relationships among several breeds of pigs and Japanese wild boars based on the restriction endonuclease cleavage patterns of mtDNA. The numbers indicate the estimated percentage sequence differences among them.

Since restriction enzymes recognizing four base pairs cut far more often than six-base enzymes, the use of four-base enzymes could easily detect nucleotide sequence difference. An example employing *Taq*I is shown in Fig. 2 and Table II. mtDNAs of pigs between European and Asian groups were clearly different from each other in the *Taq*I cleavage pattern, but mtDNAs belonging in the same group had similar restriction patterns. Bottleneck effects must occur in each groups of pigs as well as in inbred laboratory mice reported by Ferris *et al.* (1982), although it is not clear when the effect occurred, either prior to or contemporary with domestication of the pig.

On the basis of blood groups and protein polymorphism, Tanaka *et al.*

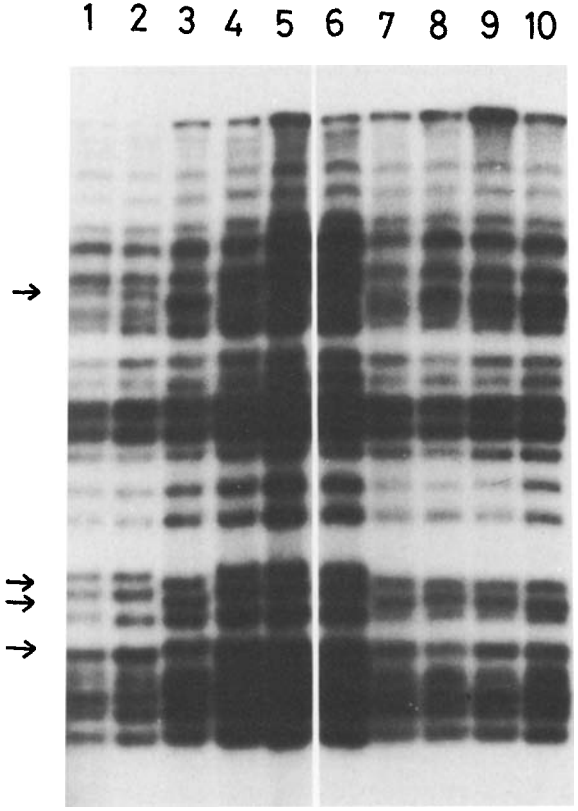

Fig. 2. Polyacrylamide gel electrophoresis of *Taq*I digests of pig mtDNA. (1) Duroc II; (2) Large White I; (3) Large White II; (4) Landrace; (5) Duroc I; (6) Duroc I; (7) Large White II; (8) Japanese wild boar; (9) Ohmini; (10) Ohmini. Samples 1, 2, 4, 5, and 6 are A types, and samples 3, 7, 8, 9, and 10 are B types. Arrows indicate the different fragments between A and B types.

Table II. Types of Restriction Endonuclease Cleavage Patterns Digested by *Taq*I and Comparison with Those of *Bgl*II, *Eco*RV, *Sca*I, and *Stu*I

Sample No.	Breeds	*Taq*I	*Bgl*II	*Eco*RV	*Sca*I	*Stu*I
1	Duroc II	A	A	B	A	A
2	Large White I	A	A	A	A	A
3	Large White II	B	B	A	B	B
4	Landrace	A	A	A	A	A
5	Duroc I	A	A	A	A	A
6	Duroc I	A	A	A	A	A
7	Large White II	B	B	A	B	B
8	Japanese wild boar	B	B	A	B	B
9	Ohmini	B	B	A	B	B
10	Ohmini	B	B	A	B	B

(1983) concluded that the European and Asian pig populations are distantly related; Taiwan native breeds and Ohmini miniature pigs are classified in the Asian group. The Göttingen miniature breed is also close to the Asian group (Oishi *et al.*, 1980), but the Pitman-Moore miniature breed is close to the European group (Oishi and Tomita, 1976). These results correspond well with the genetic similarity based on the mtDNA cleavage patterns. Kurosawa *et al.* (1983) have reported from immunogenetic studies that East–West geographical clines of some antigen frequencies are clearly observed in wild boars on the Eurasian continent (Buschman, 1965; Wiatroszka, 1970; Tikhonov *et al.*, 1972) and that wild boars in Japan are closer to Far Eastern than Middle Asian Transcausian and European boars. Wild boars on the Eurasian continent need to be investigated from the view of mtDNA polymorphism, because the study could suggest some ideas about the process of domestication and evolution of pigs.

As the restriction endonuclease map of pig mtDNA was only provisionally drawn in the previous report, position 0 was not identified in the D-loop region, unlike human, mouse, and bovine mtDNAs. Although the complete nucleotide sequences of human, mouse, and bovine mtDNAs have already been determined (Anderson *et al.*, 1981, 1982; Bibb *et al.*, 1981), no sequence data have been reported yet for pig mtDNA. Sequence data could make a precise restriction endonuclease map orientation by means of the homology test with human, mouse, and bovine mtDNAs. Pig mtDNA has four *Hind*III cleavage sites, so that four cleavage fragments, of 9.2, 4.3, 2.5, and 0.5 kb, are obtained. The two *Hind*III cleavage fragments of 4.3 and 2.5 kb from Pitman-Moore were cloned into the *Hind*III site of M13mp10 and were sequenced by the dideoxynucleotide-chain termination method. A clone with a 4.3-kb fragment inserted has the nucleotide sequence of the terminal as shown

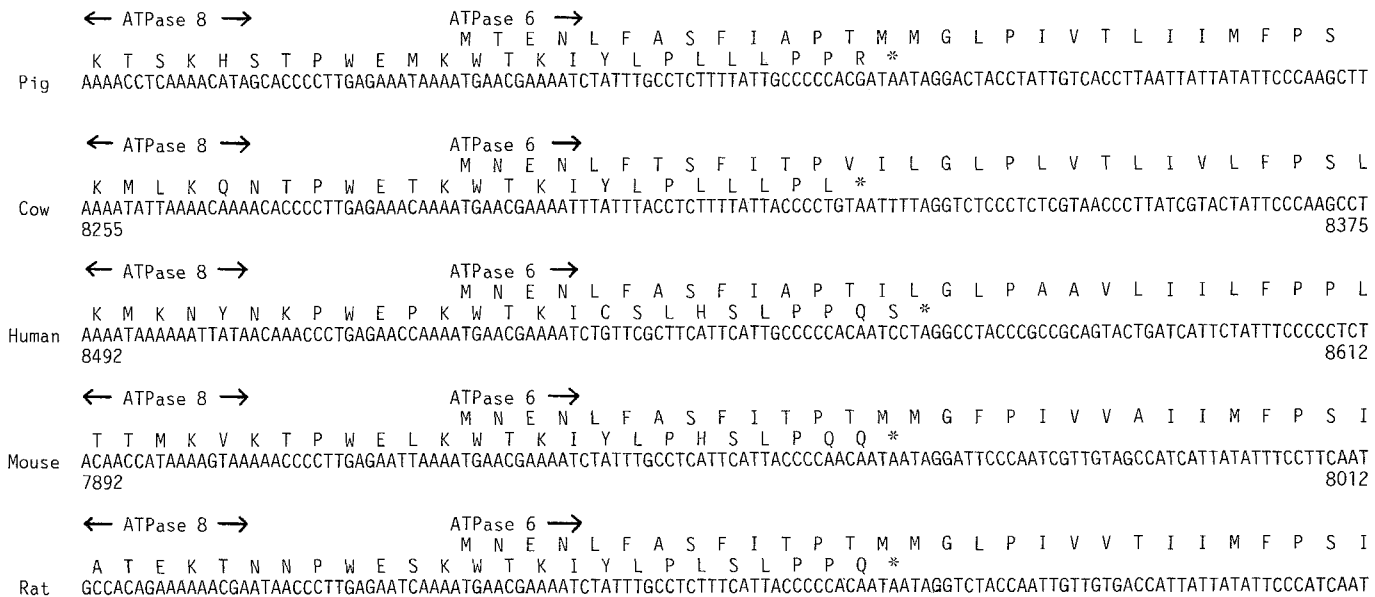

Fig. 3. The nucleotide sequence of the terminal in a clone containing a *Hind*III cleavage fragment of 4.3 kb inserted into M13mp10 and the homology test with cow, human, mouse, and rat mtDNAs.

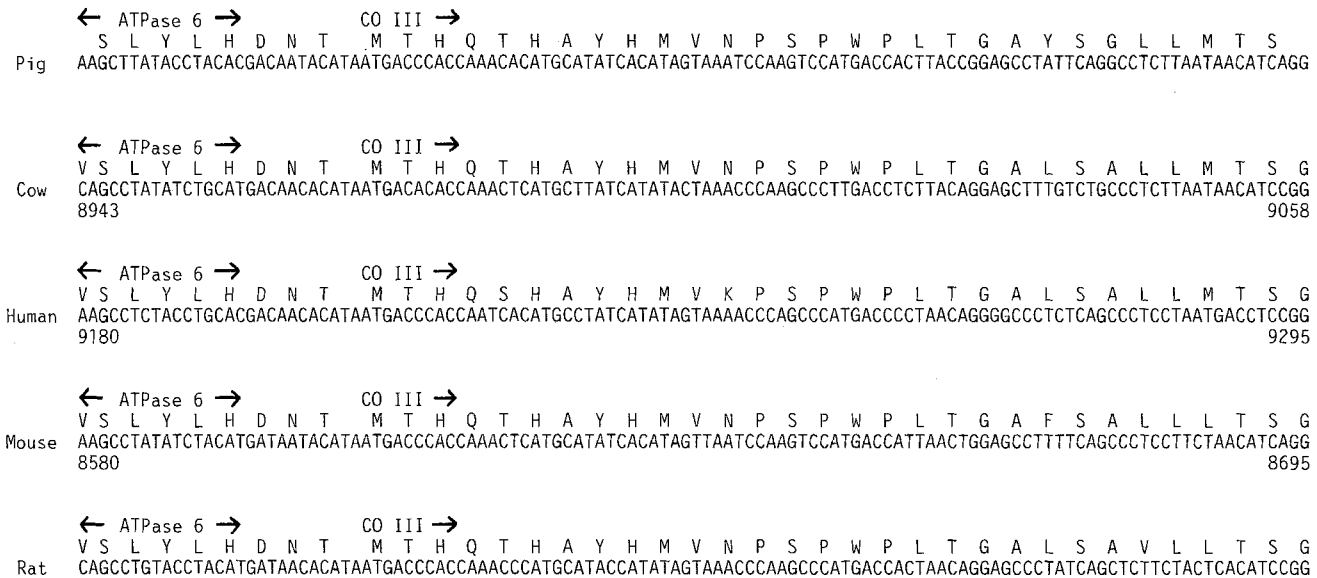

Fig. 4. The nucleotide sequence of the terminal in a clone containing a *Hind*III cleavage fragment of 2.5 kb inserted into M13mp10 and the homology test with cow, human, mouse, and rat mtDNAs.

in Fig. 3. The nucleotide sequence of the terminal of the other clone including a 2.5-kb fragment is presented in Fig. 4. The sequences of both fragments were identified to correspond to a part of the ATPase subunit by analysis of alignment with the sequence data of other mammals. With these data we have assigned position 0 of pig mtDNA in the D-loop region in Fig. 5. Furthermore, *Dra*I and *Stu*I cleavage sites on the map were newly determined in this study. On the basis of these results, the restriction endonuclease map of pig mtDNA was oriented as shown in Fig. 5. Up to here, polymorphic sites were not determined to be located in any genes. The A type has three *Bgl*II cleavage sites in regions 12 S RNA, CoII, and URF5, but the B type has only one site in the CoII region. The A type in *Eco*RV has an *Eco*RV cleavage site in URF5, but the B type has two sites in the 16 S RNA and URF5 regions. The polymorphic sites for *Stu*I must be located in URF4L and Cytb; a cleavage

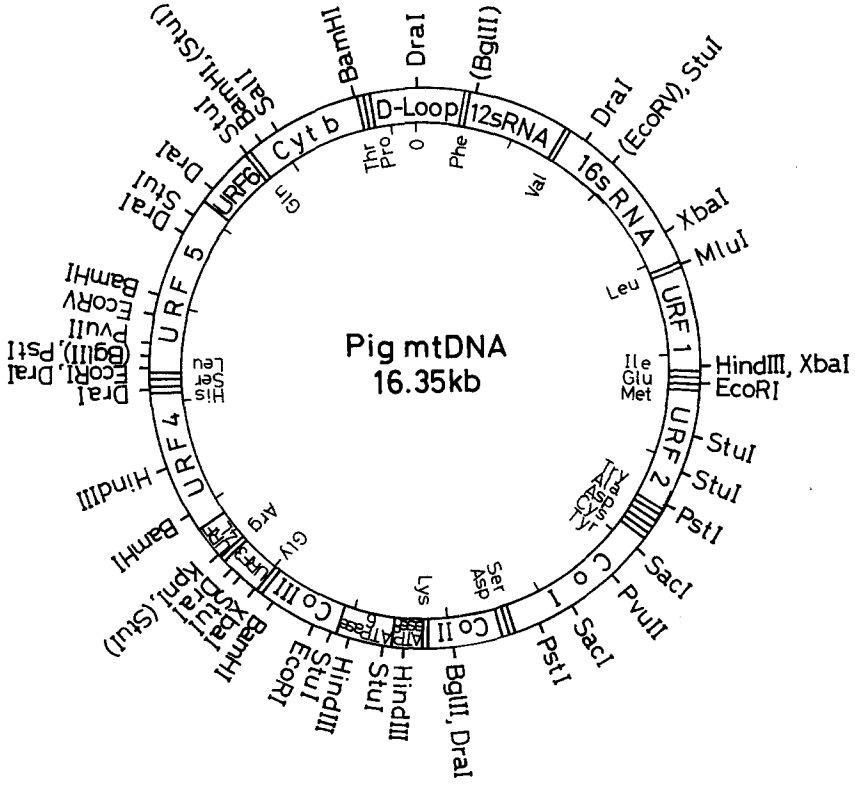

Fig. 5. The restriction endonuclease cleavage map of pig mtDNA. Position 0 must be in the D-loop region and ATPase 6 is located between position 8.43 and position 8.92. Restriction endonucleases in parentheses show the different polymorphic sites among pigs.

Table III. Sequence Differences Among Mammalian Mitochondrial DNAs at 237 Positions[a]

	Pig	Cow	Human	Mouse	Rat
Pig	—	47	61	46	51
Cow	19.8	—	60	60	59
Human	25.7	25.3	—	62	57
Mouse	19.4	25.3	26.2	—	41
Rat	21.5	24.9	24.1	17.3	—

[a]The number of sequence differences between any two mtDNAs appears in the upper right-hand section of the matrix, and the percentage sequence difference is given in the lower left-hand section.

site of the A type is in the URF4L region and not in the Cytb region and that of the B type is in the Cytb region and not in the URF4L region.

As the mtDNA sequence of pig mtDNA is available also in the rat (Pepe *et al.*, 1983), the sequence differences among five species of mammalian mtDNAs were compared at 237 positions (Table III). It would be reasonable that the number of differences between the mouse and the rat, which are both rodents, is the least. Of interest is that the number of differences between the pig and the cow is almost the same as that between the pig and the mouse and between the pig and the rat. Pigs and cows might be distantly related, although both species are artidactyl, and the pig is considered to be closer than the cow to rodents. As shown in Table IV, the number of nucleotide substitutions was counted after dividing them into silent and replacement changes and transitions and transversions. With respect to the number of replacements and transversions, the differences between the pig and the cow are indeed smaller than between any other pairs except the mouse and the rat.

Table IV. The Number of Nucleotide Substitutions Among Mammalian Mitochondrial DNAs at 237 Positions

Species compared	Silent	Replacement	Transition	Transversion
Pig–cow	25	22	24	23
Pig–human	24	37	32	29
Pig–mouse	21	25	19	27
Pig–rat	21	30	22	29
Cow–human	26	34	32	28
Cow–mouse	30	30	22	38
Cow–rat	27	32	29	30
Human–mouse	22	40	27	35
Human–rat	21	36	27	30
Mouse–rat	23	18	23	18

More sequence data for pig mtDNA will be necessary to discuss the relationships among mammalian mtDNAs in detail.

ACKNOWLEDGMENTS

We would like to express our thanks to Dr. R. Semba of the Institute for Developmental Research for his useful discussions and his kind guidance on how to analyze the sequence data with a hand-type personal computer.

We also thank Mr. H. Ohmi of the Nippon Research Laboratory for Domestic Animals and Drs. M. Mitsui and M. Mizutani of the Nippon Institute for Biological Science for making the animals available for this study.

REFERENCES

Anderson, S., Bankier, A. T., Barrell, B. G., de Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, P. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981). Sequence and organization of the human mitochondrial genome. *Nature* **290**:457.

Anderson, S., de Bruijn, M. H . L., Coulson, A. R., Eperon, I. C., Sanger, F., and Young, I. G. (1982). The complete sequence of bovine mitochondrial DNA: Conserved features of the mammalian mitochondrial genome. *J. Mol. Biol.* **156**:683.

Bibb, M. J., Van Etten, R. A., Wright, C. T., Walberg, M. W., and Clayton, D. A. (1981). Sequence and gene organization of mouse mitochondrial DNA. *Cell* **26**:167.

Brown, W. M., George, M., Jr., and Wilson, A. C. (1979). Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. USA* **76**:1967.

Brown, W. M., Prager, E. M., Wang, A., and Wilson, A. C. (1982). Mitochondrial DNA sequences of primates: Tempo and mode of evolution. *J. Mol. Evol.* **18**:225.

Buschmann, H. (1965). Blood group serological studies. In *Proceedings of the 9th European Conference on Animal Blood Groups,* Prague, 1964, p. 131.

Ferris, S. D., Sage, R. D., and Wilson, A. C. (1982). Evidence from mtDNA sequences that common laboratory strains of inbred mice are descended from a single female. *Nature* **295**:163.

Groneborn, B., and Messing, J. (1978). Methylation of single-stranded DNA in vitro introduces new restriction endonuclease cleavage sites. *Nature* **272**:375.

Kurosawa, Y., Oishi, T., Tanaka, K., and Suzuki, S. (1979). Immunogenetic studies on wild pigs in Japan. *Anim. Blood Groups Biochem. Genet.* **10**:227.

Haring, F., Gruhn, R., Smidt, D., and Scheven, B. (1965). Miniature swine development for laboratory purposes. In *Swine in Biomedical Research,* Battelle North West, Washington, p. 789.

Messing, J., Crea, R., and Seeburg, P. H. (1981). A system for shotgun DNA sequencing. *Nucleic Acids Res.* **9**:309.

Nei, M., and Li, W. -H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proc. Natl. Acad. Sci. USA* **76**:5269.

Oishi, T., and Tomita, T. (1976). Blood groups and serum protein polymorphisms in the Pitman-Moore and Ohmini strains of miniature pigs. *Anim. Blood Groups Biochem. Genet.* **7**:27.

Oishi, T., Esaki, K., and Tomita, T. (1980). Genetic relationship among Göttingen miniature, European and East Asian pigs investigated from blood groups and biochemical polymorphism. *Jap. J. Zootech. Sci.* **51**:226.

Pepe, G., Holtrop, M., Gadaleta, G., Kroon, A. M., Cantatore, P., Gallerani, R., De Benedetto,

C., Quagliariello, C., Sbisà, E., and Saccone, C. (1983). Non random patterns of nucleotide substitutions and codon strategy in the mammalian mitochondrial genes coding for identified and unidentified reading frames. *Biochem. Int.* **6**:553.

Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**:5463.

Sanger, F., Coulson, A. R., Barrell, B. G., Smith, A. J. H., and Roe, B. A. (1980). Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J. Mol. Biol.* **143**:161.

Tanaka, K., Oishi, T., Kurosawa, Y., and Suzuki, S. (1983). Genetic relationship among several pig populations in East Asia analyses by blood groups and serum protein polymorphisms. *Anim. Blood Groups Biochem. Genet.* **14**:191.

Tikhonov, V. N., Gorelov, I. G., and Troshina, A. I. (1972). Immunogenetic studies of wild European, Asian and American Suiformes in connection with the origin of some antigens in *Sus scrofa domestica*. *Anim. Blood Groups Biochem. Genet.* **3**:173.

Wakana, S., Watanabe, T., Hayashi, Y., and Tomita, T. (1986). A variant in the restriction endonuclease cleavage pattern of mitochondrial DNA in the domestic fowls (*Gallus gallus domesticus*). *Anim. Genet.* (in press).

Watanabe, T., Hayashi, Y., Ogasawara, N., and Tomita, T. (1985a). Polymorphism of mitochondrial DNA in pigs based on restriction endonuclease cleavage patterns. *Biochem. Genet.* **23**:105.

Watanabe, T., Mizutani, M., Wakana, S., and Tomita, T. (1985b). A demonstration of maternal inheritance of avian mitochondrial DNA in chicken-quail hybrids. *J. Exp. Zool.* **236**:245.

Watanabe, T., Hayashi, Y., Semba, R., and Ogasawara, N. (1985c). Bovine mitochondrial DNA polymorphism in restriction endonuclease cleavage patterns and location of the polymorphic sites. *Biochem. Genet.* **23**:947.

Wiatroszak, I. (1970). Studies on blood groups in wild boar. In *Proceedings of the 11th European Conference of Animal Blood Groups and Biochemical Polymorphism,* Warsaw, 1968, p. 265.