Annals of Warsaw University of Life Sciences – SGGW
Animal Science No 49, 2011: 27–34
(Ann. Warsaw Univ. of Life Sc. – SGGW, Anim. Sci. 49, 2011)

# Utilization of the selected microsatellite sequences in optimizing the mating plan in an experimental flock of Ayam Cemani breed

JOANNA GRUSZCZYŃSKA[1], MONIKA ŁUKASIEWICZ[2], BEATA GRZEGRZÓŁKA[1]
[1]Department of Genetics and Animal Breeding, Warsaw University of Life Sciences – SGGW
[2]Section of Poultry Breeding, Department of Animal Breeding, Warsaw University of Life Sciences – SGGW

**Abstract:** *Utilization of the selected microsatellite sequences in optimizing the mating plan in an experimental flock of Ayam Cemani breed*. The parental flock initially included 2 cocks and 9 hens, that originated from different breeding farms, and then the following $F_1$ generation included 42 birds (15 hens and 27 cocks). The experimental flock of Ayam Cemani breed was bred at the Poultry Farm of the University of Life Sciences in Wilanów-Obory. Owing to the specific character of the experiment, the following microsatellite sequences were selected based on literature data: MCW0145, MCW0184, MCW0210, LEI0071, and ADL0306, all being linked with the body weight of hens (Atzmon et al., 1998; Atzmon et al., 2008; Weissmann et al., 1998; Tatsuda and Fujinaka, 2001). The earlier investigations demonstrated that the microsatellite sequences investigated in chicken of the Ayam Cemani breed were polymorphic (Gruszczyńska and Łukasiewicz, 2010). Results of molecular analyses enabled determining the genetic distance between all pairs of birds in the flock. It was demonstrated that the chosen microsatellite sequences might be successfully used as genetic markers in the tests concerning chicken origin. The method involving the use of microsatellite sequences in order to determine the genetic distance and then to optimize the mating plan turned out to be successful, for in the experimental flock the hatchability from set eggs increased to a highly significant extent from 50.1% to 74.1%.

*Key words*: Ayam Cemani breed, microsatellite sequences, genetic distance, mating plan optimization.

## INTRODUCTION

Ayam Cemani is a rare black breed of chickens originating from Indonesia. It was first time imported into Europe in 1998 by a Dutch breeder Jan Steverink. The population of Ayam Cemani in Europe is small, they are usually bred as ornamental chickens.

The choice of polymorphic *loci*, suitable for genetic analysis must be preceded by many tests on the ground of which the level of heterozygosity ($H_E > 0.6$ and PIC > 0.6), allele frequency, mutation frequency, the possibility of correct genotyping as well as a risk of committing the errors will be, *inter alia*, determined. Microsatellite sequences are short tandem repeats (STRs), consisting of two-, three-, and four-nucleotide motives. They are usually located in the non-coding regions of the genome. They are commonly used in the molecular analysis of animal origin. The polymorphic DNA microsatellites are preferred markers in pedigree control in human as well as in many animal species (Freis et al., 1990; Bowling et al., 1997; Jamieson and Taylor, 1997; Schnabel et al., 2000; Fung et al., 2002; Ganai and

Yadav, 2005; Wenk et al., 2005; Oliveira et al., 2006; Radko, 2008; Riojas-Valdes et al., 2009). The microsatellite sequences are also used in studies addressing the reconstitution of species, as well as in order to determine the genetic relationship between animals and to perform matings that would assure the greatest genetic diversity in a herd and prevent homozygosity increase in the population.

## MATERIAL AND METHODS

### Animal material and molecular analysis

The experimental flock of Ayam Cemani breed was bred at the Poultry Farm of the University of Life Sciences in Wilanów-Obory. Initially, the flock included 2 cocks and 9 hens originating from different breeding farms (from 1 German breeder: 1♂ and 5♀, and from 1 Dutch breeder: 1♂ and 4♀). The obtained offspring generation included 42 birds (15 hens and 27 cocks). In the first year of the study, results of the hatchability of the imported birds were not satisfactory, which might have been due to high homozygosity between the birds. Hence, an attempt was made to construct a mating plan for the experimental flock of Ayam Cemani breed, based on the specified genetic diversity. Following the plan, 13 pairs (at the age of 1 year) were mated and kept in separate pens throughout the reproductive period. The eggs obtained were incubated in weekly sets of 60 eggs.

The method used to determine the genetic diversity of the investigated population of birds included 5 microsatellite sequences (MCW0145, MCW0184, MCW0210, LEI0071, ADL0306). Owing to the specific character of the experiment, the microsatellite sequences were chosen based on literature data, that in chicken were linked with the body weight (Atzmon et al., 1998; Atzmon et al., 2008; Weissmann et al., 1998; Tatsuda and Fujinaka, 2001). The earlier research demonstrated that microsatellite sequences analyzed in the investigated flock of Ayam Cemani breed were polymorphic (Gruszczyńska and Łukasiewicz, 2010). The values obtained for expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), and Polymorphic Information Content (PIC) confirm the usability of the selected microsatellite sequences as markers being a convenient tool in the genetic diversity analysis of a chicken population (Gruszczyńska and Łukasiewicz, 2010).

### Statistical analysis of results

Based on the results of the previous study (Gruszczyńska and Łukasiewicz, 2010): the frequency of alleles and genotypes, expected heterozygosity ($H_E$), observed heterozygosity ($H_O$), Polymorphic Information Content (PIC), and the exact test of Hardy-Weinberg equilibrium were calculated with the Cervus 3.03. Programme (Kalinowski et al., 2007). Additional determinations were made for the probability of exclusion (PE) and combined probability of exclusion (CPE) (Wenk et al., 2005) and genetic distance between the individuals (Nei, 1972):

1. The a priori probability of exclusion PE was obtained using the formula by Evett and Weir (1998):

   $$PE = (H_O)^2 [1 - 2 H_O (1 - H_O)^2]$$

   where $H_O$ is the observed frequency of heterozygotes in database of the offspring group (n = 42) and the whole population (n = 53).

2. The Combined exclusion probability (CPE) was calculated using the formula (Jamieson and Taylor., 1997; Wenk et al., 2005):

$$CPE = 1 - (1 - PE1)(1 - PE2)(1 - PE3)(1 - PE4)(1 - PE5)$$

3. Nei's standard genetic distance enabling the determination of the genetic diversity between pairs of individuals originating from the common founder population, was computed using the following formula (Nei, 1972):

where $f_{Fi}$ and $f_{Bi}$ denote frequencies of the *i*-th allele in respectively *F* and *B* population.

$$d_{FB} = -\ln\left(\frac{\sum_i f_{Fi} f_{Bi}}{\sqrt{\sum_i f_{Fi}^2 \sum_i f_{Bi}^2}}\right)$$

The results achieved were analyzed with the use of a matrix of distances determined between particular males and females based on the frequency of alleles occurring in the 5 analyzed *loci*.

The values obtained for all combinations of pairs enabled optimizing the mating plan. In this way, pairs were made of birds with possibly the highest value of the genetic distance.

## RESULTS AND DISCUSSION

It was found that the number of alleles, varying from 4 to 8, the level of $H_E$ and PIC value (above 0.7) and the probability to identify two individuals with the same genotype 1:2721600 (Tab. 1), as indicated by Crooijmans et al. (1996), Crooijmans et al. (1997), Rosario et al. (2009), Weissmann et al. (1998), Tadano et al. (2007), are sufficiently high to apply the selected microsatellite sequences in checking the origin of the birds derived from the assumed parents. In turn, in the analyzed flock of Ayam Cemani, the probability of finding two birds with the same genotype using these sequences reached 1:6480 (Tab. 1).

Table 2 collates values of the exact test of Hardy-Weinberg equilibrium in terms of the investigated microsatellite sequences in the parental and $F_1$

TABLE 1. Number of possible genotypes in a domestic chicken and in the investigated flock of Ayam Cemani breed

| *Locus* | Number of alleles in domestic chicken | Number of possible genotypes* | Number of alleles in Ayam Cemani | Number of possible genotypes* |
|---|---|---|---|---|
| MCW0145 | 8 [a),e)] | 36 | 4 | 10 |
| MCW0184 | 6 [b)] | 21 | 2 | 3 |
| MCW0210 | 4 [b), d)] | 10 | 3 | 6 |
| LEI0071 | 8 [c)] | 36 | 3 | 6 |
| ADL0306 | 4 [d)] | 10 | 3 | 6 |
| Probability of finding two individuals with the same genotype | | 1:2721600 | | 1:6480 |

[a)] Crooijmans et al., 1996; [b)] Crooijmans et al., 1997; [c)] Rosario et al., 2009; [d)] Weissmann et al., 1998; [e)] Tadano et al., 2007.

* following to the formula: {n!/[2(n – 2)!]}+2, where n – number of alleles.

generation as well as for the entire analyzed population of Ayam Cemani breed. It was demonstrated that in the case of the MCW0210 microsatellite sequence the differences in genotypes distribution were significant, whereas in the case of the ADL0306 sequence – highly significant (Tab. 2).

The computed coefficient of the probability of exclusion (PE) and combined probability of exclusion (CPE), accounting for 0.973 in the parental group and 0.981 in the offspring group (Tab. 3) of the investigated flock, point to the feasibility of applying the selected microsatellite sequences for origin determination in the experimental flock of Ayam Cemani breed.

Based on the frequency of the selected microsatellite sequences, coefficients of observed heterozygosity ($H_O$), expected heterozygosity ($H_E$) and Polymorphic Information Content (PIC) were computed in the analyzed population. The calculated values of $H_E$ coefficient were ranging from 0.48 to 0.71 in the parental generation and from 0.39 to 0.64 in the $F_1$ generation (Tab. 3). Values of the PIC were slightly lower than those of the $H_E$. The highest number of homozygotes in both generations was observed for the MCW0184 sequence, i.e.: 82% and 81%, respectively. In terms of the ADL0306 sequence, none homozygotes were identified in the parental generation, whereas in the offspring generation they constituted 4.8%. In addition, in the $F_1$ generation, the loss of 218bp allele was observed in the MCW0145 *locus*.

Due to the fact that the initial number of birds (parental generation) consisted of 11 birds (2 cocks and 9 hens) and that the origin of the offspring generation was not completely identified, all birds in the flock were genotyped. Owing to the fact that based on the results achieved the origin of the birds was still not inexplicit, use was made of the values of a standard genetic distance computed for each pair of birds based on the frequency of alleles. The mean genetic distance in the parental generation (11 birds) accounted for 0.4877, whereas in the offspring generation (42 birds) – for 0.5432. Values of the mean genetic distance of particular males with a group of females and that of individual females with a group of males were reported in Table 4. The birds were mated so as the genetic distance between

TABLE 2. Results of the exact test of Hardy-Weinberg equilibrium in terms of the analyzed microsatellite sequences in the experimental population of Ayam Cemani breed

| Locus | MCW0145 | MCW0184 | MCW0210 | LEI0071 | ADL0306 |
|---|---|---|---|---|---|
| Parental generation | ND | ND | ND | ND | ND |
| Offspring generation | NS | ND | ND | NS | *** |
| The whole investigated population of Ayam Cemani | NS | ND | * | NS | *** |

Exact test of Hardy-Weinberg equilibrium:
* significant differences ($p \leq 0.05$)
** highly significant differences ($p \leq 0.01$)
*** extremely significant differences ($p \leq 0.001$)
NS – not significant.
ND – not determined owing to a too few number of groups.

TABLE 3. Values of coefficients of: observed heterozygosity ($H_O$), expected heterozygosity ($H_E$), Polymorphic Information Content (PIC), probability of exclusion (PE) and combined probability of exclusion (CPE) in the experimental flock of Ayam Cemani breed

| Locus | Length of allele in Ayam Cemani (bp) | Values of coefficients in parental generation (n = 11) | | | | Values of coefficients in offspring generation (n = 42) | | | | Values of coefficients in the whole experimental population (n = 53) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | $H_O$ | $H_E$ | PIC | PE | $H_O$ | $H_E$ | PIC | PE | $H_O$ | $H_E$ | PIC | PE |
| MCW0210 | 156;166; 168 | 0.727 | 0.593 | 0.504 | 0.471491212 | 0.643 | 0.449 | 0.355 | 0.3456849 | 0.660 | 0.481 | 0.399 | 0.3691309 |
| MCW0184 | 240; 246 | 0.182 | 0.485 | 0.356 | 0.025056281 | 0.190 | 0.391 | 0.312 | 0.0270996 | 0.189 | 0.410 | 0.323 | 0.0201670 |
| LEI0071 | 280; 290;300 | 0.818 | 0.567 | 0.436 | 0.632863592 | 0.452 | 0.527 | 0.408 | 0.1488406 | 0.528 | 0.530 | 0.414 | 0.2131973 |
| MCW0145 | 206; 212; 214; 218*) | 0.727 | 0.710 | 0.615 | 0.471491212 | 0.738 | 0.548 | 0.460 | 0.4894614 | 0.736 | 0.592 | 0.514 | 0.4861220 |
| ADL0306 | 119;121; 125 | 1.00 | 0.602 | 0.484 | 1.0 | 0.952 | 0.637 | 0.553 | 0.9023281 | 0.962 | 0.642 | 0.564 | 0.9228729 |
| Mean | | 0.6908 | 0.5914 | 0.479 | CPE = 1.0 | 0.595 | 0.5104 | 0.4176 | CPE = 0.9729813 | 0.615 | 0.531 | 0.4428 | CPE = 0.9807237 |

*) there is no 218bp allele in the offspring generation.

TABLE 4. Mean genetic distance between individual males and a group of females and between individual females and a group of males in the experimental flock of Ayam Cemani breed

| No of male | 693 | 694 | 695 | 696 | 697 | 698 | 699 | 700 | 701 | 702 | 703 | 704 | 705 | 706 | 707 | 708 | 709 | 710 | 711 | 712 | 713 | 714 | 715 | 716 | 717 | 718 | 719 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Females | 0.3344 | 0.5216 | 0.5095 | 0.5201 | 0.2818 | 0.9642 | 0.5350 | 0.3600 | 0.5306 | 0.3600 | 0.3724 | 0.3616 | 0.4295 | 0.3416 | 0.9790 | 0.5261 | 0.7665 | 0.3616 | 0.3415 | 0.7665 | 0.7958 | 0.7665 | 0.7665 | 0.7665 | 0.5178 | 0.5306 | 0.3600 |
| No of female | 678 | 679 | 680 | 681 | 682 | 683 | 684 | 685 | 686 | 687 | 688 | 689 | 690 | 691 | 692 | | | | | | | | | | | | |
| Males | 0.4983 | 0.7764 | 0.4918 | 0.4260 | 0.4170 | 0.6744 | 0.5033 | 0.4980 | 0.4882 | 0.4703 | 0.5785 | 0.4472 | 0.8027 | 0.6335 | 0.4430 | | | | | | | | | | | | |

the pairs of mated individuals was as high as possible.

In the first phase (before optimizing the mating plan) of hatching the eggs originating from one-year-old hens of the $F_1$ generation, despite a satisfactory fertility at a level of 91%, the hatchability of set eggs reached as little as 50.0% (N = 60). The major reason behind that was a high embryonic death rate – at each developmental stage. The conducted bacteriological examinations did not reveal any health disorder of the hens. The likely cause could, thus, be high homozygosity of the mated individuals and misadaptation to a new environment. Once the mating plan had been optimized based on the computed genetic distances, no significant changes were observed in the egg fertility (92%), whereas a highly significant ($p \leq 0.01$) increase was noted in hatchability of set eggs (N = 270) to a level of 74.1%. As reported by Borzemska and Kosowska (1997), losses in hatchings reaching from 7.5% to 20%, depending on the species, breed and utilization of birds, should be considered normal, i.e. within the physiological range. According to Borzemska (2005), in the case of hens the hatchability reaches 80–92%, and even 93.5%. However, an earlier study by this author (Borzemska, 1996) suggests that the hatchability of 78% may point to nutritional errors or wrong flock matching. A high diversity in the body weight of birds observed in the experimental flock of Ayam Cemani (1320 g to 2192 g), may significantly affect the hatchability as well as the quality of chicks. This in turn results in non-simultaneous (desynchronized) hatching of chicks, i.e. early from small eggs and late from large eggs.

This manuscript presented the feasibility of applying microsatellite sequences for identifying birds origin and then for optimizing the mating plan.

## CONCLUSIONS

1. The selected microsatellite sequences might be successfully used as genetic markers in the tests concerning chicken origin.

2. The method involving the use of microsatellite sequences in order to determine the genetic distance and then to optimize the mating plan turned out to be successive, for in the experimental flock the hatchability from set eggs increased to a highly significant extent from 50.1% to 74.1%.

## REFERENCES

ATZMON G., BAXTER-JONES C., YONASH N., CHENG H., AVIDAN N., LAVI U., CAHANER A., HILLEL J., 1998: Microsatellite markers associated with quantitative traits in broilers. Proceedings, 10th European Poultry Conference" The Poultry Industry Towards the 21st Century", Jerusalem Israel, 21–26 June, 1: 191–194.

ATZMON G., BLUM S., FELDMAN M., CAHANER A., LAVI U., HILLEL J., 2008: QTLs detected in a multigenerational resource chicken population. The J. Hered. 99 (5): 528–538.

BORZEMSKA W.B., 1996: Embriopatologia drobiu. Drobiarstwo, 10: 5–9.

BORZEMSKA W.B., 2005: Choroby drobiu. Praca zbiorowa pod redakcją M. Mazurkiewicza. Wyd. Akad. Rol. we Wrocławiu, 69–124.

BORZEMSKA W.B., KOSOWSKA G., 1997: Ważniejsze problemy w patologii lęgów u drobiu. Zesz. Nauk. Przegl. Hod. 3: 25–31.

BOWLING A.T., EGGLESTON-STOTT M.L., BYRNS G., CLARK R.S., DILEANIS S., WICTUM E., 1997: Validation of microsatellite markers for routine horse parentage testing. Anim. Genet. 28: 247–252.

CROOIJMANS R.P., DIJKHOF R.J.M., POEL J.J., GROENEN M.A.M., 1997: New microsatellite markers in chicken optimized for automate fluorescent genotyping. Anim. Genet. 28: 427–437.

CROOIJMANS R.P., VAN OERS P.A., STRIJK J.A., VAN DER POEL J.J., GROENEN M.A., 1996: Preliminary linkage map of the chicken (*Gallus domesticus*) genome based on 77 microsatellite markers: 77 new markers mapped. Poultry Sci. 75: 746–754.

EVETT L.W., WEIR B.S., 1998: Interpreting DNA Evidence: Statistical Genetics for Forensic Scientists. Sinauer Associates. Inc., Sunderland. MA.

FREIS R., EGGEN A., STRANZIENGER G., 1990: The bovine genome contains polymorphic microsatellites. Genomics 8: 403–440.

FUNG W.K., CHUNG Y., WONG D., 2002: Power of exclusion revisited: probability of excluding relatives of the true father from paternity. Int J.Legal Med 116: 64–67.

GANAI N.A., YADAV B.R., 2005: Parentage determination in three breeds of Indian goat using heterologous microsatellite markers. In: H.P.S. Makkar and G.J. Viljoen (eds.) Applications of Gene-Based Technologies for Improving Animal Production and Health in Developing Countries 613–620.

GRUSZCZYŃSKA J., ŁUKASIEWICZ M., 2010: Microsatellite polymorphism in the study of genetic diversity of an experimental flock of Ayam Cemani breed, Ann. Warsaw Univ. of Life Sci. – SGGW, Anim. Sci. 47: 39–44.

JAMIESON A., TAYLOR ST.C.S., 1997: Comparisons of three probability formulae for parentage exclusion. Anim. Genet. 28: 397––400.

KALINOWSKI S.T., TAPER M.L., MARSHALL T.C., 2007: Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16: 1099–1006.

NEI M., 1972: Genetic distance between populations. The American Naturalist. 106: 283–291.

OLIVEIRA S., TRINDADE-FILHO A., MENDES C., PAULA K., MAIA F., PAK H., DALTON G., 2006: Power of exclusion of 18 autosomic STR loci in a Brazilian Middle-West region population sample. International Congress Series 1288: 433–435.

RADKO A., 2008: Ocena polimorfizmu markerów mikrosatelitarnych DNA wykorzystywanych w kontroli rodowodów bydła w Polsce. Wiadomości Zootechniczne, R. XLVI, 4: 3–8.

RIOJAS-VALDES V.M., GOMEZ DE LA FUENTE J.C., GARZA-LOZANO J.M., GALLARDO-BLANCO D.C., DE TELLITU_SCHUTZ J.N., WONG-GONZALEZ A., DAVALOS-ARANDA G., SALINAS-MELENDEZ J.A., 2009: Exclusion probabilities of 8 DNA microsatellites in 6 cattle breeds from Northeast Mexico. J. of Anim. and Vet. Advences 8(1): 62–66.

ROSARIO M.F., LEDUR M.C., MOURA A.S.A.T., COUTINHO L.L., GARCIA A.A.F., 2009: Genotypic characterization of microsatellite markers in broiler and layer selected chicken lines and their reciprocal F1s. Sci. Agric. (Piracicaba, Braz.) 66: 150–158.

SCHNABEL R.D., WARD T.J., DERR J.N., 2000: Validation of 15 microsatellites for parentage testing in North American bison, Bison bison and domestic cattle. Anim. Genet. 31: 360–366.

TADANO R., NISHIBORI M., NAGASAKA N., TSUDZUKI M., 2007: Assessing genetic diversity and population structure for commercial chicken lines based on forty microsatellite analyses, Poultry Sci. 86: 2301–2308.

TATSUDA K., FUJINAKA K., 2001: Genetic mapping of the QTL affecting body weight in chickens using a F2 family. Br. Poultry Sci. 42(3): 333–337.

WEISSMANN S., CARMON T., DOUAIRE M., CAHANER A., ZEITLIN G., LAVI U., AVIDAN N., LECLERCQ B., HILLEL J., 1998: Marker gene frequencies within and between divergently selected lines for high and low abdominal fat in chickens. Proceedings, 10th European Poultry Conference" The Poultry Industry Towards the 21st Century", Jerusalem Israel, 21–26 June, 1: 280–283.

WENK R.E., GJERTSON D.W., CHIAFARI F.A., HOUTZ T., 2005: The specific power of parentage exclusion in a child's blood relatives. Transfusion 45: 440–444.

**Streszczenie:** *Wykorzystanie wybranych sekwencji mikrosatelitarnych w optymalizacji planu kojarzeń doświadczalnego stada kur rasy ayam cemani*. Stado rodzicielskie liczyło

początkowo 2 koguty i 9 kur, które pochodziły z różnych hodowli, a następne uzyskane po nich pokolenie $F_1$ liczyło 42 ptaki (15 kur i 27 kogutów). Stado doświadczalne pochodziło z fermy drobiu Rolniczego Zakładu Doświadczalnego SGGW Wilanów-Obory. Ze względu na specyfikę prowadzonego doświadczenia na podstawie literatury wybrano sekwencje mikrosatelitarne: MCW0145, MCW0184, MCW0210, LEI0071, ADL0306, które były u kur związane z masą ciała (Atzmon et al., 1998; Atzmon et al., 2008; Weissmann et al., 1998; Tatsuda and Fujinaka, 2001). We wcześniej przeprowadzonych badaniach stwierdzono, że wybrane sekwencje mikrosatelitarne u kur rasy ayam cemani są polimorficzne (Gruszczyńska and Łukasiewicz, 2010). Na podstawie wyników badań molekularnych wyznaczono dystans genetyczny między wszystkimi parami ptaków występujących w stadzie. Stwierdzono, iż wybrane sekwencje mogą być z sukcesem wykorzystywane w identyfikacji pochodzenia u kur. Metoda wykorzystania sekwencji mikrosatelitarnych w celu ustalenia dystansu genetycznego, a następnie na tej podstawie ustalenia planu kojarzeń okazała się trafna, gdyż w doświadczalnym stadzie wylęgowość z jaj nałożonych wzrosła wysoko istotnie statystycznie z 50,1% do 74,1%.

*MS. received September 2011*

Authors' addresses:
**Joanna Gruszczyńska, Beata Grzegrzółka**
Katedra Genetyki i Ogólnej Hodowli Zwierząt – SGGW
ul. Ciszewskiego 8
02-786 Warszawa
Poland
e-mail: joanna_gruszczynska@sggw.pl

**Monika Łukasiewicz**
Katedra Szczegółowej Hodowli Zwierząt – SGGW
ul. Ciszewskiego 8
02-786 Warszawa
Poland